Age-related alterations in the level and expression pattern of Von Willebrand Factor (VWF)

by

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Abstract

Von Willebrand Factor (VWF) is a multimeric adhesive glycoprotein that is exclusively expressed in endothelial cells (EC) and megakaryocytes. This procoagulant protein is involved in maintaining primary hemostasis and thrombus formation. However, there are pathological and physiological conditions that alter the circulating levels and pattern of VWF expression in vasculature. Unregulated increase in VWF levels may contribute to elevate the incidence of thrombosis. Since increased thrombogenicity is observed with aging, we explored whether aging is associated with alterations in the level and/or pattern of VWF expression.

This study compared circulating VWF levels in the blood of young and aged mice and rats, using ELISA. Additionally, immunofluorescence confocal microscopy, Western blot analyses, and RT-PCR analyses were used to determine VWF expression pattern, cellular protein and mRNA levels. The expression pattern of the VWF protein was determined in combination with the endothelial marker CD31 and micro vessels marker (Isolectin-GS-IB4), in various organs of young and aged mice. Furthermore, to explore the functional consequences of altered VWF expression with regard to platelet aggregation, which is a major contributing factor to thrombus formation, CD41 a marker of activated platelets was used for immunofluorescence confocal microscopy.

The results of these analyses demonstrated that circulating VWF protein levels were higher in blood of aged rats and mice compared to young. Also with age VWF expression at mRNA levels were significantly increased in livers, brains, and lungs, but not in hearts and kidneys. Consistent with the mRNA analyses, the cellular protein levels were determined in lungs and livers and shown to be significantly increased in aged compared to young mice. Moreover, the endothelial staining for VWF was observed significantly in microvessels of brains, lungs, and livers of aged but not young mice, demonstrating a phenotypic shift of microvascular endothelial cells of these organs with aging towards a procoagulant state with de novo expression of VWF. Additionally, activated platelet aggregates and occluded vessels were significantly increased in livers, brains, and lungs, but not in hearts and kidneys of aged mice compared to young, consistently with the mRNA and protein levels, and redistribution patterns of VWF expression.

These results demonstrate that with aging, VWF levels are increased in circulation, and vasculature of distinct organs exhibit an altered VWF expression pattern. Specifically in liver, lung, and brain, but not heart and kidney, with aging increased VWF levels and de novo expression in microvascular endothelial cells are observed. Furthermore, the increased VWF levels specifically in these organs were concomitant with detection of significantly increased platelet aggregate formation and occluded vessels. Overexpression of VWF in circulation and microvasculature vessels of distinct organs as a result of aging may contribute to vascular diseases such as thrombosis.

Preface

The information in this thesis is the original work performed by Radya Yousef Abdualla. Experimental design, performance and analysis of data were completed by Radya Yousef Abdualla and her supervisor Dr. Nadia Jahroudi.

Dedication

"The aim of science is, on the one hand, as complete a comprehension as possible of the connection between perceptible experiences in their totality, and, on the other hand, the achievement of this aim by employing a minimum of primary concepts and relations." – Albert Einstein

I dedicate this to my family, for their great support and love throughout this journey.

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Faraj, my dear brother, I am here today because of you. Thank you for your support and patient.

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Glossary

ABO	Blood Group System
ADAMTS13	A Disingtegrin And Metalloproteinase with a Thrombospondin Type 1 motif, member 13
ADP	Adenosine Diphosphate
ALI	Acute Lung Injury
APS	Ammonium Persulfate
aPTT	Activated Partial Thromboplastin Time
BMC	Basement Membrane Components
BSA	Bovine Serum Albumin
CD31	Cluster of Differentiation 31
CD41	Cluster of Differentiation 41
cDNA	Complementary Deoxyribonucleic Acid
CHD	Coronary Heart Disease
CK	Cysteine-Knot
CVD	Cardiovascular Disease
DAPI	4',6-Diamidino-2-Phenylindole
ddH_2O	Double Distilled Water
ECM	Extra Cellular Matrix
ECs	Endothelial Cells
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay

ER	Endoplasmic Reticulum
Ets	E-Twenty-Six
FVIII	Factor VIII
GATA 6	GATA-Binding Factor 6
GFR	Glomerular Filtration Rate
GPIb-α	Glycoprotein Ib Alpha Chain
HEK293	Human Embryonic Kidney cells
HIF1α	Hypoxia-Inducible Factor 1-Alpha
HLP	Histone-Like Protein
HMTs	Histone Methyltransferases
HMW	High Molecular Weight
HPRT	Hypoxanthine-Guanine Phosphoribosyl Transferase
HUVEC	Human Umbilical Vein Endothelial Cells
IB4-GS	Isolectin Grifonnia Simplicifolia
ICAM	Intracellular Adhesion Molecule
IF	Immunofluorescence
IU	International Unit
KB	Kilo Base G
KD	Kilo Dalton
mRNA	Messenger RNA
NaOH	Sodium Hydroxide
NFI	Nuclear Factor I
NFY	Nuclear Transcription Factor Y

NO	Nitric Oxide
NP-40	Nonyl phenoxypolyethoxylethanol
OCT	Optimal Cutting Temperature Compound
PAF	Platelet Activating Factor
PAI	Plasminogen Activator Inhibitor
PBS	Phosphate-Buffered Saline
PECAM-1	Platelet Endothelial Cell Adhesion Molecule-1
PFA	Paraformaldehyde
pH	Potential Of Hydrogen
PVDF	Polyvinylidene fluoride
RBF	Renal Blood Flow
RNA	Ribonucleic Acid
RT-PCR	Real-Time Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
SNGFR	Single Nephron Glomerular Filtration Rate
TBS-T	Tris-Buffered Saline -Tween 20
TEMED	Tetramethylethylenediamine
TF	Transcription Factors
TFPI	Tissue Factor Pathway Inhibitor
TGN	Trans Golgi Network
Tie	Tyrosine Kinase With Immunoglobulin-Like And EGF-Like Domains
TTP	Thrombotic Thrombocytopenic Purpura

UL-VWF	Ultralarge Von Willebrand Factor Multimers
VD	Vascular Dementia
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Cell-Specific Receptor
VWD	Von Willebrand Disease
VWF	Von Willebrand Factor
VWF-Ag	VWF antigen
VWFpp	VWF Propeptide
WB	Western Blot
WPB	Weibel-Palade Bodies

CHAPTER 1: INTRODUCTION

1. Von Willebrand Factor (VWF)

1.1. VWF structure

Von Willebrand Factor (VWF) is a multimeric adhesive glycosylated protein that circulates in plasma and is expressed exclusively in endothelial cells (ECs) and megakaryocytes as a primary translation product of 2,813 amino acids. This highly cell type restricted expression pattern of VWF is used as a marker to distinguish endothelial cells from other cell types [1- 3].

The human *VWF* gene is located on the short arm of chromosome 12, and it contains 52 exons [4]. The primary sequence of the VWF gene was reported in 1987 [5]. The *VWF* gene is transcribed into a 9 kb mRNA, which is translated into a protein of 2813 amino acids with an estimated MW of 310 kD [4].

The synthesis of the VWF is a complex multistep process, which generates a pre-pro-polypeptide containing 22 amino acids signal peptide, 741 amino acids pro-polypeptide (propeptide), and the VWF mature subunit [4]. The 22 amino acids signal sequence is cleaved co-translationally [6], and the 741 amino acids propolypeptide is removed at a later step from the amino terminus resulting in the mature VWF protein of 2050 amino acids that is extensively glycosylated [4,6]. The post-translational processing of VWF includes

dimerization, glycosylation, sulfation, propeptide cleavage, and multimerization, followed by storage or secretion [5,7].

VWF architecture is a mosaic of four different domains that are repeated two to four times each [4]. There are three A-domains, three B-domains, two C-domains and four D-domains. The B-domains are small and contain 25 to 35 amino acid residues, while the duplicated C-domains contain 116 to 119 residues. The four D-domains contain 351 to 376 residues present in four copies. These are arranged in the following sequence: D1-D2-D′-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK (Figure 1) [8]. For each domain, specific functions and interactions with different proteins have been reported. For instance, the D′-D3 domains bind to factor VIII, the A1 domain binds to the platelet receptor glycoprotein (GP) Ibα, and contains additional binding sites for heparin and sulfated glycolipids. The A2 domain contains the cleavage site for the metalloprotease ADAMTS-13, which is a VWF cleaving enzyme. Both A1 and A3 domains bind to extracellular matrix (ECM) proteins (collagen, fibronectin) in the endothelial basement membrane and connective tissues [8,9,10]. The C1 domain is the binding site for the integrin αIIbβ3 on platelets surface [4].

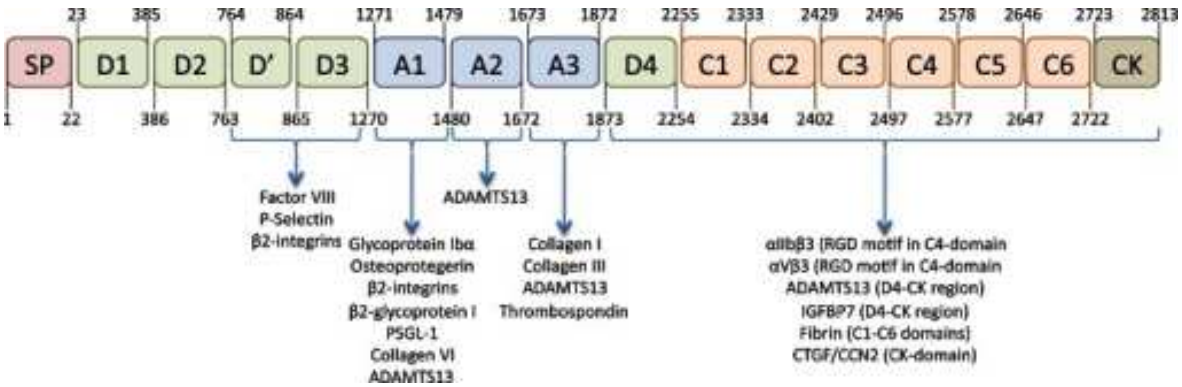


Figure 1: Schematic representation of the VWF domain architecture and location of interactive sites [8].

In the endoplasmic reticulum (ER), pro-VWF assembles into dimers (also called

protomers) at the carboxyl-terminal end in a "tail-to-tail" fashion by forming inter-chain disulfide bonds between cysteine-knot (CK) domains, which are the cysteine-rich regions of the VWF, and mediate the formation of disulfide-linked dimers [11]. In the trans-Golgi network (TGN), dimers of pro-VWF assemble into multimers by forming "head-to-head" inter-chain disulfide bonds between D3 domains at the amino termini, and subsequently, the propeptide is cleaved by furin to generate high-molecular weight (HMW) multimers consisting of up to 100 subunits [11,12,13]. The modifications in the trans-Golgi network include the proteolytic removal of the large VWF propeptide, multimer formation, glycosylation and sulfation. Multimerization depends on the decrease in pH between the ER (pH 7.2) and TGN (pH 6.2) [14], and the VWF propeptide plays an essential role in the assembly of multimers, since it was shown that deletion of the propeptide abolishes the multimerization [4,15,16]. The VWF propeptide is cleaved and the multimeric, mature VWF is packed and stored in rod-shaped organelles known as Weibel-Palade bodies (WPBs) in ECs [16,17,18], and stored in the alpha granules in platelets [19,20]. Stored VWF is thus comprised of the largest multimers, also referred to as Ultra-Large VWF (UL-VWF), which is usually not observed in a significant amount in circulation under normal conditions [8,21,22]. The second storage site for VWF is within the platelet α-granules, which may contribute to approximately 20% of the total VWF present in blood [4]. The VWF of platelet α-granules also consists of the ultra-large VWF multimers [23,24]. Upon stimulation stored VWF are released into circulation and the pro-peptide dissociates from VWF multimers and circulates independently as a non-covalent homodimer with a very short half-life of 2 hours. The plasma level of VWF propeptide is 1 μg/mL. In contrast, mature VWF is secreted in the plasma as oligomers containing a variable number of subunits, ranging from a minimum of two subunits to a maximum 40 subunits, with the

largest multimers having molecular weights of more than 20 kD. VWF multimers are cleared slowly with a half-life of 12 hours, and the plasma concentration averages 10 µg/mL [4]. The complex intracellular pathway involved in biosynthesis and organization of VWF are depicted below in Figure 2 [21].

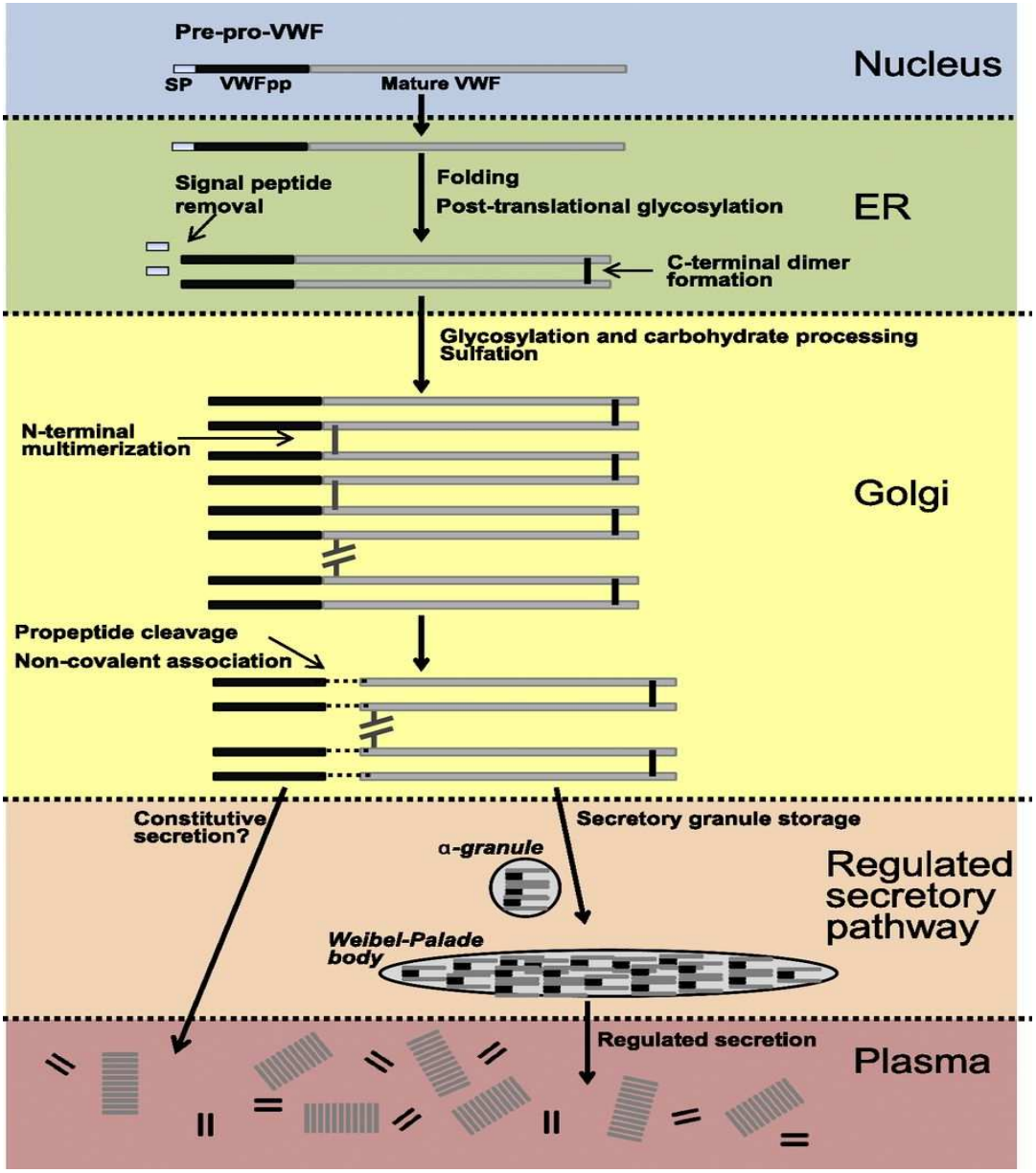


Figure 2: **VWF intracellular processing.** VWF gene is transcribed as mRNA coding for pre-pro-VWF (nucleus, blue) and the translated product is translocated to the ER (green), where it

undergoes signal peptide removal, glycosylation, and dimer formation. In the Golgi (yellow) O-linked glycans are added, carbohydrates are processed, VWF is sulfated and multimerized, and propeptide is cleaved from mature VWF but remains noncovalently associated. VWF is trafficked to the regulated secretory pathway (peach) and stored in WPBs or α-granules before release into plasma (red), where VWFpp and VWF dissociate and circulate independently of one another. Dimer or smaller multimers of VWF can also be released directly from cytoplasm into circulation through constitutive pathway [18].

1.2. VWF gene and promoter, and mechanism of VWF transcription

VWF gene is located on chromosome 12, and is comprised of 52 exons [25], of which the first exon that is 250 nucleotides long, entirely codes for 5' untranslated region of the mRNA. Beginning of exon 2 corresponds to translation initiation codon methionine. VWF transcription is regulated by a complex mechanism [28,29], which has been explored through analysis and identification of the VWF promoter [30]. Our group demonstrated that nucleotides −487 to +247 of the VWF gene function as an endothelial-specific promoter in cultured cells and transgenic mice [26,27].VWF gene expression regulation is a complex system that includes activation of transcription machinery through interaction of transacting factors with specific DNA cis-acting elements in the promoter and other DNA regulatory regions [30,31]. Transacting factors that interact with the VWF promoter function either as activators, such as GATA, ETS, NFY, and HLP (Histone H1-like protein), or repressors, such as NFI, OCT, NFY and E4BP4, of the VWF promoter activity [25,29,30]. The NFY

transcription factor functions as both an activator and a repressor of the VWF promoter activity, based on its two distinct binding sequences (Figure 3) [29]. NFY functions as an activator when interacting with the sequence CCAAT, which is its consensus binding site at position −18 in the VWF promoter; whereas it functions as a repressor when interacting with a non-consensus binding sequence corresponding to nucleotides +226 to +234 in the first exon [26,30,31].

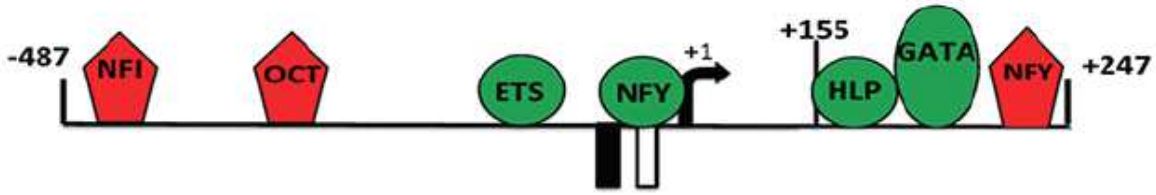


Figure 3: Schematic representation of the VWF promoter region and transcription factors that positively (green) and negatively (red) regulate the promoter activity [29]. **Transcription start site is shown by arrow marked as +1. The solid bar represents TATA element and the open bar represents the CCAAT element.**

1.3. VWF Function

VWF plays critical roles in initiating primary hemostasis (stops the escape of blood from vessels). The primary function of VWF is to promote platelet binding to the sub endothelial tissue at the site of a vascular injury. VWF also mediates platelet adhesion to ECs surfaces

and promotes platelet–platelet interactions, thus contributing to thrombogenicity. A second critical role for VWF is that it serves as the carrier protein for coagulation factor VIII (FVIII), protecting it from proteolytic degradation in plasma, thus stabilizing factor VIII, and extending its half-life in circulation [4,32]. However, in addition to these long-known functions of VWF it has recently been shown that VWF plays important roles in other pathophysiological and physiological processes as well, such as inflammation, tumour metastasis, angiogenesis and cell proliferation (**References**). Thus, it is not surprising that dysregulated levels of VWF, whether increased or decreased, contribute to many diseases [29,33].

As discussed above, the VWF functions by mediating the adherence of platelets to each other and to the surfaces of ECs / sub ECs. This process is mediated through interaction of specific VWF domains with the glycoproteins GPIbα, and GPIIb/IIIa on the surface of platelets [17]. The binding of VWF to these complexes facilitates the activation and aggregation of platelets and their interaction with ECs' basement membrane components (BMC), or adherence to the ECs' luminal surface [32]. (Figure 4) [34]. These actions are important in the formation of a blood clot that stops bleeding and maintains hemostasis. However, it can also contribute to unwanted thrombogenicity if unregulated. Additionally, the interaction between factor VIII and VWF is necessary for normal survival of blood clotting factor VIII in blood circulation to participate in appropriate hemostasis [6, 34, 35]. Thus, to prevent unwanted clot formation, VWF generally under normal conditions circulates in low concentrations [35].

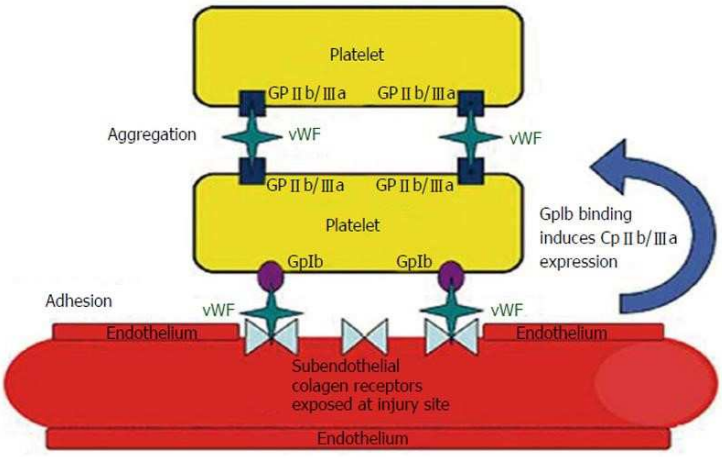


Figure 4: The role of Von Willebrand factor in the platelet adhesion and platelet aggregation [34].

Normally circulating UL-VWF molecules are folded (unevenly compacted on itself) due to self-association between VWF monomers, which prevent exposure of the active binding sites [36]. However, upon activation for instance by shear stress, which acts on the VWF molecule, stretching it, and changing its conformation, VWF becomes unfolded. Unfolded VWF exposes platelet-binding domains, specifically the VWF A1 domain; as well as A2 domain which contains the binding domain and the cleavage site for the VWF cleaving enzyme ADAMTS13 [36,37]. Exposure of A1 domain therefore allows VWF to bind to the glycoprotein complexes on platelets and initiate platelet plug formation, while unfolding also exposes cleavage sites for ADAMTS13, which cuts VWF into fragments that have little or no ability to bind to and activate platelets [37], thus limiting the growth and maintenance of the platelet plug formation.

UL-VWF is stored in storage organelles, and not present in circulation in significant

amounts. However, in response to stimuli VWF is secreted into circulation. The stimuli may include injury or various external stimuli such as proinflammatory or prothrombotic stimuli, interleukins, hypoxia, and radiation among others [29]. VWF is secreted via constitutive or regulatory pathways [6]. While dimer or small multimer forms of VWF protein exist in the cytoplasm and are released constitutively from endothelial cells, extra-large multimers of VWF protein are exocytosed from WPBs upon endothelial cell activation or released from injured endothelial cells [38].

VWF expression pattern and VWF abnormalities

The distribution patterns of the VWF protein through vasculature reflect endothelial cell heterogeneity [26,27]. Endothelial cells from veins and arteries and from capillaries of different organs exhibit heterogeneity at structural, functional and molecular levels. Similarly, there are differences in VWF expression levels in veins, arteries, and capillaries and among vessels of similar size in distinct organs [26,39]. Even though VWF molecules in WPBs are organized similarly, the sizes, shapes and number of WPBs are also different according to the cytoplasmic space of endothelial cells of distinct vascular beds. The cells that have smaller cytoplasmic space also have a smaller size and a fewer numbers of WPBs [40].

The production of VWF must be under regulation because the unregulated VWF production can lead to low or high levels of this protein, which in either case can contribute to various disease processes [41]. Decreased levels or defective function of VWF cause the most common inherited bleeding disorder known as Von Willebrand Disease (VWD) [42].

Additionally, the mutational changes in VWF domains induce functionally deficient forms of the VWF protein, which have been identified in various types of Von Willebrand Disease [43]. A quantitative deficiency of VWF is a diagnostic feature of VWD type 1 (Type 1 VWD), which is characterized by low levels of VWF [44,45]. However, qualitative deficiencies of VWF that are known as Type II VWD, may result from mutations that affect its structural integrity, multimerization, or binding efficiency to its target molecules such as Factor VIII [46]. In circulation, VWF binds non-covalently to factor VIII, thus mutations that interfere with VWF interaction with FVIII lead to a marked decrease in plasma FVIII levels and a significant increase in the "activated partial thromboplastin time (aPTT), a measure of contact-phase-dependent coagulation [47]. VWF deficiency might occur due to defects at any point in VWF biosynthesis pathway, and / or due to mutational changes in VWF domains [44,48]. Low VWF is a diagnostic feature of Type 1 VWD, which is characterized by partial quantitative deficiency of VWF [49,50].

On the other hand, a high level of circulating VWF is a risk factor of cardiovascular diseases such as ischemic heart disease [51], thrombogenicity, and increased mortality in the general population [52]. There are many pathological and physiological conditions that increase the levels of VWF in circulation and alter the VWF expression patterns in tissues. VWF contributes to increased thrombogenicity, which may lead to cardiovascular complications. Increased plasma levels of VWF are detected in various diseases; for instance stroke [53], ischemic heart disease [54,55], hypertension [56], atherosclerosis [54,57], myocardial infarction injury [58,59], acute lung injury [60.61], thrombotic thrombocytopenic purpura (TTP) [22,62], diabetes [63], hypernatremia [64,65], inflammatory diseases [66], malaria [67] hypoxia [68], and cancers [69,70]. VWF levels

were also reported to exhibit differences according to race, with people of African decent showing VWF levels 15 to 18 % higher than those of Caucasian . [71].

In a normal population, VWF plasma levels are elevated with increasing age [13,72]. As Lillicrap et al (2015) demonstrated, circulating plasma VWF levels increases by approximately 0.17 and 0.15 IU/ml per decade and this increase could contribute to increases in cardiovascular diseases that are associated with aging [46]. However, the mechanisms of age-related increase in VWF plasma levels remain unknown [44].

2. AGING

2.1. Diseases associated with aging

Aging can be defined as the normal process of life that is characterized by changes or degeneration of organs of various systems leading to loss of their anatomical and physiological functions [66]. The global share of older people, aged 60 years or older, increased from 9.2% in 1990 to 11.7% in 2013, and will continue to grow to 21.1% by 2050 [66]. With aging, physiological changes occur in all organ systems [70]. For example, the cardiac output decreases and blood pressure increases. With aging blood vessels become stiff and less responsive to hormones, which relax the vessels' valves, and thus result in increased systolic blood pressure [71]. Arteriosclerosis development in elderly is another major contributing factor to cardiovascular disease, which is the primary cause of mortality in the Western world [72,73]. In addition to rise in cardiovascular disease, the respiratory system is also significantly affected by aging. The lungs show impaired gas

exchange, a decrease in vital lung capacity, and lower expiratory flow rates. With aging, lungs become rigid due to loss of elasticity, thus ventilation and gas exchange are affected, resulting in decreased respiratory flow [70]. Other systems that are target of age related increase in disease/reduced optimal function include central nervous system and gastrointestinal, and renal systems. Age associated alterations in central nervous system and its associated vasculature contribute to decline in cognitive function, dementia, Alzheimer disease, and stroke, which are well known causes of increased mortality and morbidity in elederly (Ref?) [74]. In the elderly, the stomach also shrinks due to mucosal inflammation that is called atrophic gastritis, and the stomach produces insufficient amount of acid due to lack of vitamin B12. Therefore, the colon becomes hypotonic, resulting in constipation [70]. The size and function of the kidney begins to decrease after forty years of age and significantly decreases by sixty years [75], resulting in decreased levels of creatinine clearance, although the serum creatinine level remains relatively constant [70]. Regardless of the system/organ, vascular damage, specifically increased thrombogenicity, is one of the major contributing factors to dysfunction of various organs. Increased thrombogenicity in the elderly contributes to vascular dementia (VD), stroke, CVD, and atherosclerosis (Ref?). [57].

The aging process is associated with increased vascular rigidity and vascular damage due to proliferation and consequently accumulation of smooth muscle cells and connective tissue in the walls of major blood vessels [76]. Generally, to determine the correlation of vascular damage to thrombogenicity, the degree of vascular damage in relation to stability of formed thromi have been studied according to the degree of stenosis and to the severity of damaged vessel wall [59]. There are many pathological conditions and diseases, such as

inflammatory diseases, that lead to endothelial cell activation and stimulate platelet attachment to the vessels walls [58]. The degree of platelet deposition and thrombus formation after vascular damage is differentiated by type of injury and the location of damaged vessels [61].

It has been documented that when vascular wall injury is mild, the thrombogenicity is limited, resulting in transient thrombotic occlusion; whereas deep vessel injury results in relatively persistent thrombotic occlusion. Also, the platelet deposition is directly related to the degree of vascular injury. Therefore, high thrombogenicity appears when deep or severe injury to the vessel's wall occurs [59].

In normal conditions platelets, do not adhere to the intact, non-activated ECs. However, high levels of pro-inflammatory and pro-thrombotic mediators such as VWF in the circulation activate ECs and allow platelet recruitment under high shear stress [57]. Elevated levels of VWF contribute to increased thrombogenicity in some organs for instance the lung [66]. Injuries such as cerebral injury can cause a significant elevation of plasma VWF, which could lead to sustained platelet adhesion to the vessels' walls [56].

2.2. Thrombogenicity in aging

Contribution of increased thrombogenicity to vascular dysfunction, thus underscore its correlation to age-associated cardio- and cerebro- vascular diseases. Approximately, one in four deaths worldwide is attributable to stroke or ischemic heart disease. Stroke is the

leading cause of disability [2]. Venous thromboembolic disease, pulmonary embolism, and deep venous thrombosis are common coagulation diseases. The incidence of arterial and venous thrombosis increases with age [2]. A recent study has shown that the impact of thrombotic disorders worldwide is expected to increase dramatically with aging. Likewise, the risk of bleeding in the older population is also higher [77]. Thus VWF, as a central player in the processes of thrombus formation, is a major participant in determining vascular disease progression with aging.

Besides its role in thrombus formation, VWF also plays a role in inflammatory processes. VWF, in addition to fibrinogen, is an independent risk factor for development of atherosclerosis. High VWF levels are positively associated with the risk for development of coronary heart disease (CHD) and ischemic stroke (IS) [77]. VWF levels increase in acute-phase ischemic stroke because of endothelial dysfunction and this contributes to thrombus formation [2]. Elevated levels of fibrinogen, white blood cell count, and VWF are risk factors and may play causative roles in coronary heart disease. [78]

Few clinical studies have linked higher VWF levels with a greater risk of acute CHD incidence among patients with CHD [79,80]. Elevated VWF levels indicate endothelial cells dysfunction and vascular inflammation. The elevated VWF levels promote platelet adhesion to damaged arterial walls, but may also enhance platelet aggregation under sheer stress in intact micro vessels, which have high sheer stress [80,81], thus contributing to microvessels occlusion and decreased perfusion of target tissues.

In this study, we have explored the age-related alterations in VWF expression by studying the changes in VWF mRNA and protein levels, as well as the VWF expression patterns in

different organs (livers, lungs, brains, hearts, and kidneys). We have also explored the functional consequences of alteration in VWF levels by determining the presence of platelet aggregates in target organs.

2.3. VWF and aging

Although the appropriate levels of VWF are required for the hemostasis process, many genetic and environmental factors influence the levels of VWF. Age is considered among those factors. It has been demonstrated by Konkle, B. A. [13] that in human, VWF levels increase with age, and elevated VWF levels are associated with an increased risk of venous thromboembolism and cardiovascular diseases (CVD).

Patients with VWD due to a deficiency or dysfunction of VWF may have symptoms that improve with aging because they may have some protection from cardiovascular diseases (CVD). However, in those patients with VWD who develop cardiovascular diseases (CVD), management is very challenging because they cannot use the antiplatelet therapy as a treatment support [13].

Previous studies examined the change in VWF and FVIII levels with aging in patients with VWD. In Type 1 VWD, that is, patients whose VWF functions normally, but it is present in sufficient quantity, VWF and FVIII levels increase with age with no modification in bleeding phenotype. However, VWF and FVIII levels do not increase in type 2 VWD patients who have a normal VWF level, but the VWF does not work properly [45].

Increased VWF with aging may contribute to age-related vascular diseases.

As mentioned previously, high levels of VWF are associated with an increased risk for cardiovascular disease (CVD), while low levels result in prolonged bleeding. Aging is associated with both increased levels of VWF and a higher incidence of cardiovascular diseases. However, the exact mechanism responsible for this age-related increase in VWF remains unknown and a subject of this study.

CHAPTER 2: MATERIALS AND METHODS

The Health Sciences Animal Policy and Welfare Committee at the University of Alberta approved all animal housing and experimentation. All live animal manipulations, including blood collection, euthanasia, and organ removals, were performed by personnel in Dr. Stephen Bourque laboratory and according to his approved Animal Use Protocol. I performed all subsequent analyses of blood samples and organs.

Blood collection for rats from saphenous vein

1. Young (3 months) and aged (19 months) Sprague Dawley rats were used for this study.

2. The back of the hind leg was shaved with electric trimmer until saphenous vein was visible.
3. Hind leg was immobilized and slight pressure was applied gently above the knee joint.
4. Alcohol swab was used to aseptically clean the area of shaved skin.
5. The vein was punctured using an 18G needle and appropriate volume of blood (how much? 500 microliter for rats?) was collected using a syringe with a needle, and the punctured site was compressed to stop the bleeding.
6. Blood was collected into EDTA (anticoagulant)-treated tubes. For plasma preparation, cells were removed from plasma by centrifugation of the whole blood for 15 minutes at 1,000x g using a refrigerated centrifuge. The resulting supernatant (plasma) was immediately transferred into Eppendorf tubes, and stored at –20°C.

Determination of plasma VWF concentration in rat

VWF plasma concentration was determined using the Rat VWF ELISA Kit (MyBioSource, Cat # MBS005773) according to the manufacturer's protocol as follow:

1. All reagents and samples were prepared at room temperature at 30 min. before starting the experiment.
2. The samples were centrifuged after thawing before the assay.

3. Standard wells, sample wells, and blank wells were set on the microtiter ELISA plate.
4. Standards (50µl to each Standard well) and samples (50µl to each sample well) were added, and 50µl of sample diluent was added to each Blank well.
5. All Standards and samples were added in triplicate to the plate.
6. 100µl of HRP-conjugate reagent was added to each well and the plate was covered with an adhesive strip and incubate for 60 minutes at 37°C.
7. The Microtiter plate was washed four times by automated washing using wash buffer (1×). The washer was adjusted to aspirate as much wash buffer as possible at 350µl/well/wash. After final wash, the plate was inverted over absorbent paper towels for drying.
8. Chromogen Solution A (50µl) and Chromogen Solution B (50µl) were added to each well. Mixed gently, protected from light, and incubated for 15 minutes at 37°C.
9. Stop solution (50µl) was added to each well and the plate tapped gently to ensure thorough mixing. The color in the wells changed from blue to yellow.
10. The Optical Density (O.D.) was measured at 450 nm using a (Multiskan® microplate reader, Thermo Scientific) within 15 minutes after adding Stop Solution.
11. To quantify the amount VWF plasma concentrations:
    a. The averages of the triplicate readings for each standard and sample were calculated.
    b. Average optical density of the Blank subtracted.
    c. Professional curve fitting software used to make a standard curve and

calculate VWF plasma concentrations.

Blood collection and organs preparation for mice

1. Young (3 months) and aged (18 months) mice were used for this study.
2. Mice were placed in dorsal position on the table.
3. Mice were anesthetized with isoflurane (5% in 100% O2) and euthanized.
4. The skin layers and the muscles along the midline were rapidly excised.
5. Skin was flipped laterally on each side of the incision to expose the underlying tissues and organs.
6. Blood was collected by heart puncture using a 12G needle with syringe.
7. Whole blood was collected into EDTA (anticoagulant)-treated tubes.
8. Five organs including liver, brain, lung, heart, and kidney of euthanized young and aged mice were harvested and divided to three sections for following manipulations:
    a. One portion for RNA and protein analyses, which were placed in labeled cassettes, immersed in liquid nitrogen immediately, and maintained at – 80 0C.
    b. One portion for OCT immunofluorescence analysis, which were put in labeled cassettes and soaked in 30% sucrose in water overnight at 4C. Samples were then placed in OCT and frozen as described below in immunofluorescence staining section
    c. One portion for paraffin embedded immunofluorescence/immunohistochemistry analysis, which were placed in labeled cassettes and fixed in 10% formalin.

Determination of plasma VWF concentration in mice

1) For plasma preparation, the whole blood that was collected into EDTA tubes were centrifuged for 15 minutes at 1,000x g using a refrigerated centrifuge. The resulting supernatant (plasma) was immediately transferred into Eppendorf tubes, and stored at –20°C.
2) VWF mice plasma concentration was determined using the mouse VWF ELISA kit, (Elabscience®, Cat # E-EL16 M1247) according to the manufacturer's protocol as follows:
    i) All reagents and samples were brought to room temperature for 30 min. before starting the experiment.
    ii) The samples were centrifuged after thawing before the assay.
    iii) Standard wells, sample wells, and blank wells were set on the Micro ELISA plate.
    iv) The plasma samples were diluted (1:20) with sample diluent.
    v) 100µL standard or sample were added to each well. 100µl of sample diluent was added to each blank well and incubated for 90 minutes at 37 ^{O}C.
    vi) The liquid was removed and 100µL Biotinylated Detection Ab was added, followed by incubation for one hour at 37 ^{O}C.
    vii) The plate was washed three times by automated washing using wash buffer (1×). The washer was adjusted to aspirate as much wash buffer as possible at 350µl/well/wash. After final wash, the plate was inverted for drying. The excess solution was removed from wells by gently tapping the plate onto an

absorbent paper towel.

viii) 100µL HRP conjugate was added and samples were incubated for 30 minutes at 37 ^{O}C.

ix) The plate was aspirated and washed five times.

x) 90µL of substrate reagent was added to each well and incubated for 15 minutes at 37 ^{O}C.

xi) 50 µL of stop solution was added to each well. The color in the wells changed from blue to yellow.

xii) The Optical Density (O.D.) was measured immediately at 450 nm using a (Multiskan® microplate reader, Thermo Scientific).

3) To quantify the plasma VWF concentrations:

A. The averages of the triplicate readings for each standard and sample were calculated.

B. Average optical density of the Blank subtracted.

C. Professional curve fitting software was used to make a standard curve and calculate VWF plasma concentrations.

RNA preparation and real-time polymerase chain reaction (PCR) analyses

1) Real-time RT-PCR for detection and quantification of the VWF mRNA were performed on RNA prepared from frozen organs (liver, brain, lung, heart, and kidney). Total RNA was isolated from the organs using (Qiagen RNeasy plus Mini Kit Cat. # 74134) as recommended by the manufacturer.

2) RNA concentration was determined using (NanoDrop® ND1000 Spectrophotometer).

3) Two micrograms of RNA were used to generate cDNA using qScript™ cDNA SuperMix, (Quanta Biosciences, Cat. # 101414-102) as recommended by the manufacturer.

4) The cDNA was diluted (1:20) and 2 microliters were used as template for real-time PCR analysis. Real time PCR reactions were carried out with a SYBR Green Master kit (Roche Applied Science, Mannheim, Germany). The reaction contained 5 microliters of SYBR green mix, 1 microliter of each primer (forward and reverse primers), and 2 microliters of cDNA template in a 10 microliters reaction volume.

5) The PCR reactions were carried out on an ABI 7900 Genome Analyzer System (Applied Biosystem- Thermofisher). Cycling conditions used were as follows: An initial heating profile of 50°C for 2 minutes (min) and 95°C for 10 min, followed by 40 cycles of alternating cycles of 95°C for 15 seconds and 60°C for 1 min each.

6) In each experiment, VWF was normalized to the HPRT (Hypoxanthine-guanine Phosphoribosyl Transferase) and ΔCT were calculated to show the relative expression levels of VWF.

Western Blot Analysis (WB) and protein quantification

1) Lysates were prepared from frozen sections of livers, brains, lungs, hearts, and kidneys of young and aged mice using NP40 buffer as follows:
    - 1ml NP40 buffer were added to round bottom 2ml tube with one metal bead.
    - Approximately 200mg of each tissue was put in a separate eppindorf tube on dry ice.
    - Tissue grinding were performed using the tissue lyser (TissueLyser II, Qiagen, Cat #. 85300, Canada), at 30 rpm for 3 minutes or until no tissues were remained.
    - Tubes were centrifuged for 10 minutes at 12,000x g using a refrigerated centrifuge and supernatant were transferrd to new labeled tubes.
2) Total protein concentration was determined by Bradford assay using Bradford reagent, (Cat # B6916 Sigma-Aldrich). In a microtiter plate (using an eight-point dilution of BSA standard for calibration) the absorbance was detected (Multiskan® microplate reader, Thermo Scientific) at 595 nm wavelength.
3) 4X Laemmli buffer that contained β-mercaptoethanol (how much?) was added to the tissue lysate and samples were heat-denatured at 95^0C for 5 minutes. 20 to 30 microgram of proteins were loaded in each well of 6% SDS-PAGE and the gels were run for 1–2 h at 100 V.
4) The gels were transferred (using what equipment?) to PVDF membranes overnight in cold room and membranes were blocked with 5% skimmed milk (in PBS buffer) for one hour.
5) VWF protein was detected by probing the membranes with appropriate primary antibodies [1/500 Rabbit anti-VWF mouse specific antibody, Cat # ab9378, Abcam Canada), and 1/2000 mouse Anti-β-Tubulin Antibody (Cat # 05-661 –

EMD, Millipore, Canada)] diluted in blocking solution overnight in cold room.

6) Membranes were washed three times in TBST (Tris Buffered Saline Twin) 10 min each.
7) Appropriate HRP-conjugated secondary antibodies were used for detection of VWF, 1/10.000 Goat Anti-Rabbit IgG Antibody, HRP-conjugate (Cat # 12-348 – EMD, Millipore, Canada), and 1/10,000 Goat Anti-Mouse IgG Antibody, HRP conjugate, (Cat # 12-349 – EMD, Millipore, Canada).
8) Membranes were washed three times in TBST, 10 min each and developed using Amersham ECL Western Blotting Detection Reagent, (Cat # RPN2108, Canada). The excess reagents were removed and the membranes were covered in transparent plastic wrap.
9) The membranes were exposed using gel documentation system for several time-points from 30 seconds to 10 minutes.

Immunofluorescence staining

I) OCT slides preparation.

1) Tissues were collected from young and aged mice, cut into appropriate small size, and were put in labeled cassettes.
2) Tissues were soaked in 30% sucrose in water overnight at 4^0C.
3) Following sucrose treatment, tissues were washed in PBS three times and then placed into OCT, frozen over liquid nitrogen immediately and then stored at -80C until sectioning.

4) Cryosections were made using a Leica (Wetzlar, Germany CM 3050S) cryostat at 5 µm slices.
5) Sections were placed on Fisherbrand® Superfrost Plus Microscope Slides. (Cat # 1255015), and stored at -20 ^{0}C until staining.

II) Immunofluorescent staining of OCT frozen sections

1) Slides were allowed to dry, and sections were blocked with blocking buffer [containing 5% fish gelatin (5g powder fish gelatin in 100 ml PBS) and 10% serum from the host species of secondary antibody] for 1 hour at room temperature in a moisturizing container.
2) The appropriate primary antibodies were added to the specimen, overnight, in cold room. For VWF, the appropriate primary antibody was 1/200 sheep anti-VWF mouse specific antibody, □Cat # ab11713, □□□□□□□ Canada). For CD31, the appropriate primary antibody was 1/50 rabbit anti-CD31 mouse specific antibody, □Cat # ab28364, □□□□□□□ Canada). For CD41, 1/50 rat anti CD41 mouse specific, □Cat # ab33661, □□□□□□□ Canada). The primary antibodies were diluted in the blocking solution.
3) Slides were washed three times (12 min. each) in high salt PBS (1.4 gm NaCl in 500 mL 1X PBS), followed by one time 1X PBS 10 min.
4) Slides were incubated with appropriate secondary antibodies for 1 hour at room temperature. Secondary antibody for VWF was 1/1000 Donkey anti-Sheep, Alexa Fluor 488, green, (Cat # A-11015, Thermo fisher), secondary antibody for CD31 was 1/1000 Goat anti-rabbit, Alexa Fluor 594, green, (Cat # A-11037,

Thermo fisher), and secondary antibody for CD41 was 1/1000 Goat anti-rat, Alexa Fluor 568, red, (Cat # A-11077, Thermo fisher). In some experiments costaining were performed with 1/200 of conjugated Isolectin GS-IB4 antibody, (Cat # I21411, Thermo fisher). For detection of nuclei DAPI 1/1000 were added.

5) Slides were washed similarly as described in step No 3 and dried. Then the mounting media were added, and the slides were covered with coverslip and kept overnight at room temperature in a Dark place.
6) Slides were ready for confocal microscopy.

Preparation of paraffin embedded tissues

1) Tissues of young and aged mice were formalin fixed in (10% formalin) and paraffin embedded using standard protocol.
2) Tissue blocks were cut in 5 µm thickness and transferred onto microscope slides (Fisherbrand® Superfrost Plus Microscope Slides. (Cat # 1255015) followed by air-drying in room temperature for one hour.

Immunofluorescence staining of paraffin embedded tissues

1) Tissue sections were de-paraffinized and rehydrated as follows:
    a. 2 hours in 60^0C oven.

b. 3 times 10 minutes in Xylene (Fisher Scientific Cat #. AC180860010)
c. 2 minutes in 100% Ethanol
d. 2 minutes in 95% Ethanol
e. 2 minutes in 75% Ethanol
f. 2 minutes in 50% Ethanol
g. 2 minutes in running water

2) For antigen retrieval, slides were placed in 1% boiled citrate solution (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0) for 10 minutes.
3) Slides were washed with warm water and placed in PBS, and immunofluorescence staining was performed as described previously.

Statistical analyses

Data are given as mean with standard deviation (SD) and statistical analyses used was the two-tailed t test. Statistically significant changes ($p<0.05$) are marked as (*)

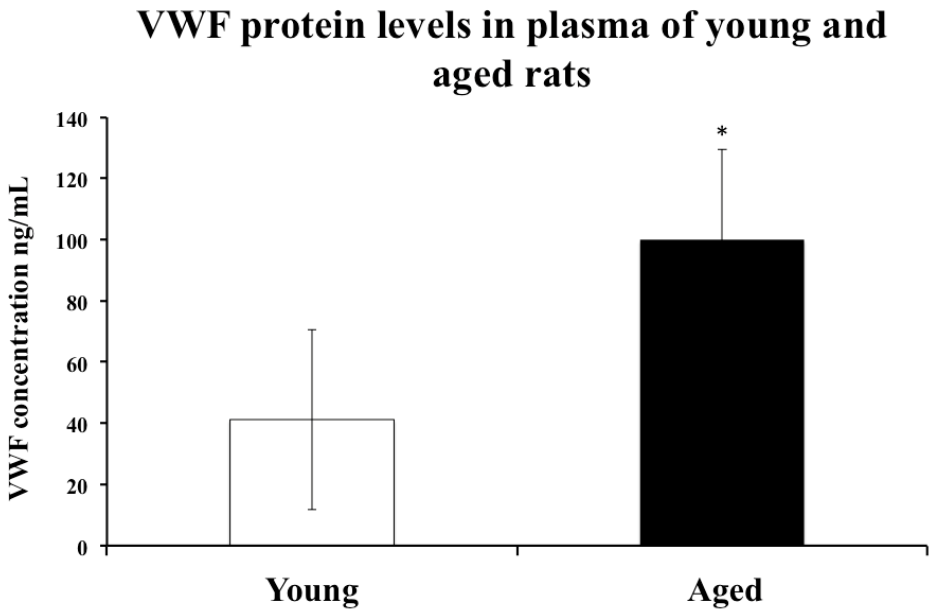
CHAPTER 3: RESULTS

1. Circulating VWF protein levels in plasma of young and aged rats and mice

VWF Protein Levels in plasma are increased in aged mice, and rats compared with young.

To test the hypothesis that VWF levels are increased with aging, we first proceeded to determine whether the levels of circulating VWF in aged and young rats and mice are different. Towards this goal, we used plasma from seven young (3 months), and seven aged (19 months) rats, and plasma from six young (3 months), and six aged (18 months) mice. The circulating VWF levels were determined using ELISA. Measuring of VWF protein levels by ELISA showed a significant increase in aged mice and rats compared to youngs (Figure. 5).

A


VWF protein levels in plasma of young and aged rats
VWF concentration ng/mL
140
120
100
80
60
40
20
0
*
Young
Aged

B

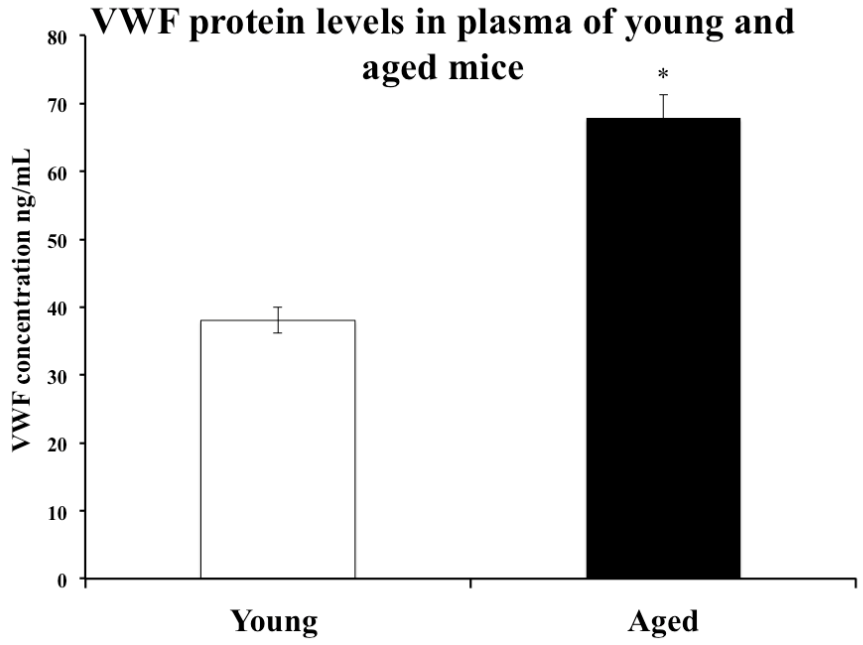

VWF protein levels in plasma of young and aged mice
VWF concentration ng/mL
80
70
60
50
40
30
20
10
0
*
Young
Aged

Figure 5: Quantitative analysis of plasma VWF by ELISA. Graphs represent concentrations of circulating VWF protein (ng/ml) in the plasma of following two groups: **(A)** Young and aged rats; and **(B)** Young and aged mice. ELISA was performed using rat and mice VWF ELISA kits as described in Materials and Methods. Results are representative of 2 independent experiments for rats (n=7) and mice (n=6) (*P<0.05), two-tailed t-test.

2. VWF mRNA levels in different organs of young and aged and mice

VWF mRNA levels increase with aging in Liver, Brain and Lung, but in heart and kidney.

Based on the previous finding, we investigated whether the differences that we had observed in the circulating VWF protein levels with aging is also reflected at VWF transcriptional levels. Towards this goal, we proceeded to determine whether VWF mRNA levels are different in various organs of aged compared to young mice, and weather such differences exhibit organ specificity. For these analyses major organs (liver, lung, brain, heart, and kidney) of six young and six aged mice were harvested, RNA was extracted, and processed for quantitative RT-PCR analyses to detect and quantify VWF mRNA and HPRT (used for normalization). The results demonstrated that there were significantly increased VWF mRNA levels in livers, brains, and lungs of aged compared to young mice. However, this change was not observed in hearts and kidneys and VWF mRNA levels in these organs of young and aged mice were similar (Figure. 6).

In livers, and lungs of aged mice, VWF mRNA Levels increased three-fold in comparison to young mice. VWF mRNA levels were four-fold increased in brains of aged mice compared to young. The results also demonstrated that there were significant differences in the levels of VWF mRNA from various organs consistent with many previous studies. This potentially represents the difference in the level of vascularization of various organs, and differential VWF expression in different vascular beds. These results not only support the hypothesis that with aging VWF mRNA is increased at transcriptional level, but also demonstrate that this process happens in an organ specific manner.

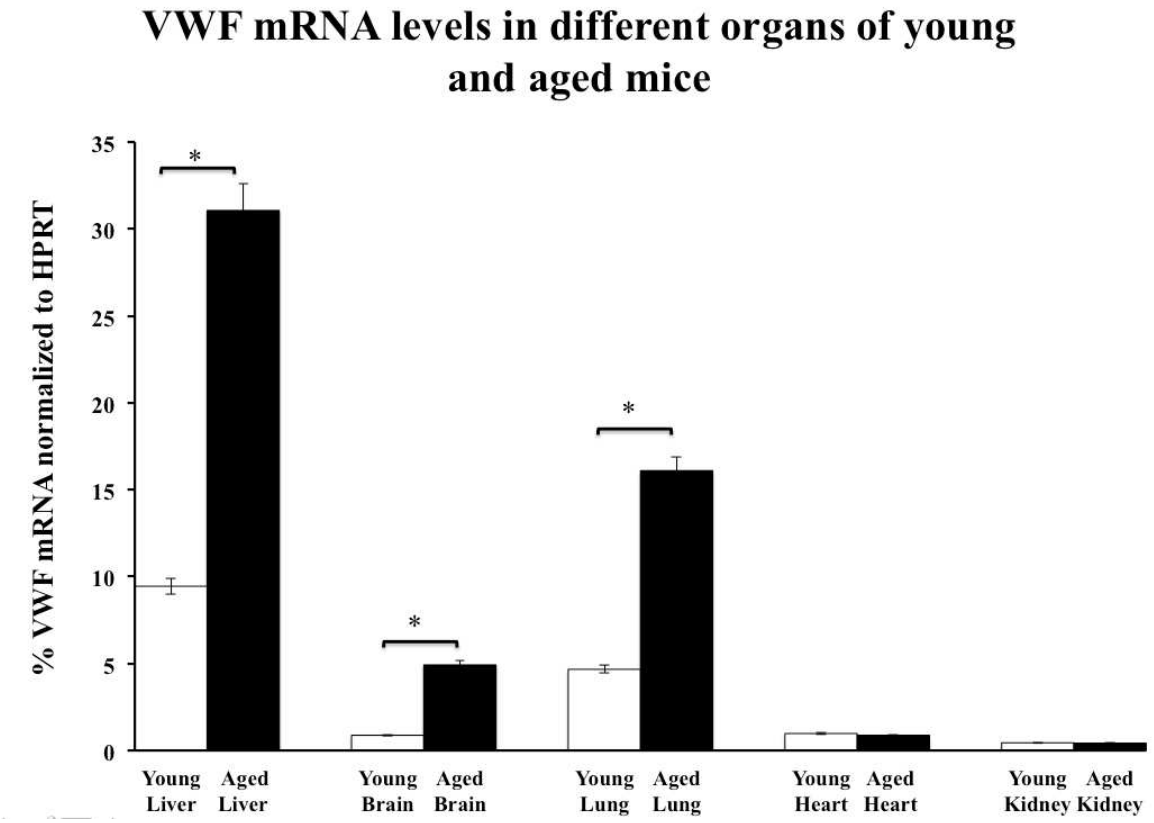


Figure 6. Quantification of VWF mRNA levels demonstrate significant differences between young and aged mice. RNA(1 μg) prepared from various organs (liver, brain, lung, heart, and kidney) of young and aged mice were subjected to reverse transcription and real-time PCR analyses to detect VWF mRNA levels. Graph represents percentage VWF mRNA normalized to HPRT in various organs (Liver, lung, brain, heart, and kidney) of young and aged mice. Results are representative of

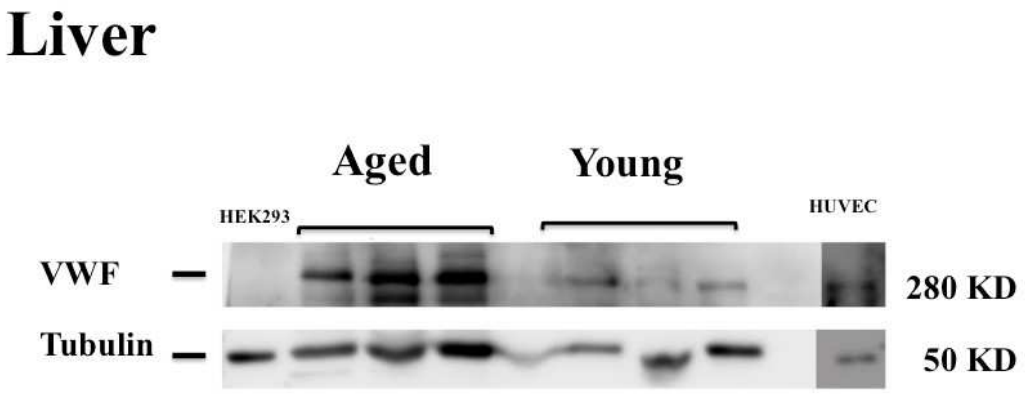
3 independent experiments from 6 different mice for each young and aged (n = 6). (*P<0.05), two-tailed t-test.

3. VWF cellular protein levels in livers and lungs of young and aged and mice.

VWF protein levels are increased in livers and lungs of aged mice compared to young

Next, we proceeded to investigate whether this increase in VWF mRNA levels correlates and translates to an increase in VWF cellular protein levels. Towards this goal, we performed Western blot (WB) analyses of total protein lysate prepared from livers and lungs of young and aged mice to determine cellular VWF protein levels. WB analyses demonstrated that VWF protein levels are significantly increased with aging in liver and lung. (Figure 7). These results are consistent with the results of RNA analyses and demonstrate that increased VWF mRNA translates into increased cellular VWF protein in target organs.

A



B

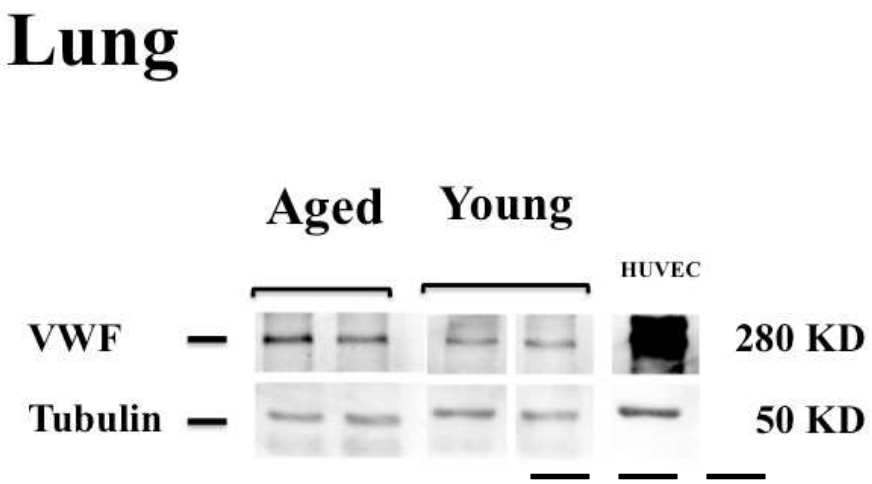


Figure 7. Representative Western blot analysis of lysates from young (3 months) and aged (18 months) mice organs. **(A)** Liver and **(B)** lung lysates from young and aged mice (20µg protein) were subjected to Western blot analysis. Total lysate (10µL, approximately 50 micro gram) from HEK293 cells, which is a VWF non-expressing cell line, was used as negative control. Total lysate (5µL, approximately 10 microgram?) from HUVEC cells, which is a VWF expressing cell type, was used as positive control for VWF expression. All samples shown were analyzed on the same gel and the black lines indicate the position where irrelevant lanes were cut out. (Representative blot of 3 independent experiments n=6).

4. VWF expression pattern in various organs of young and aged mice

Liver, lung, and, brain of aged mice increase their VWF expression and present a change in their expression pattern.

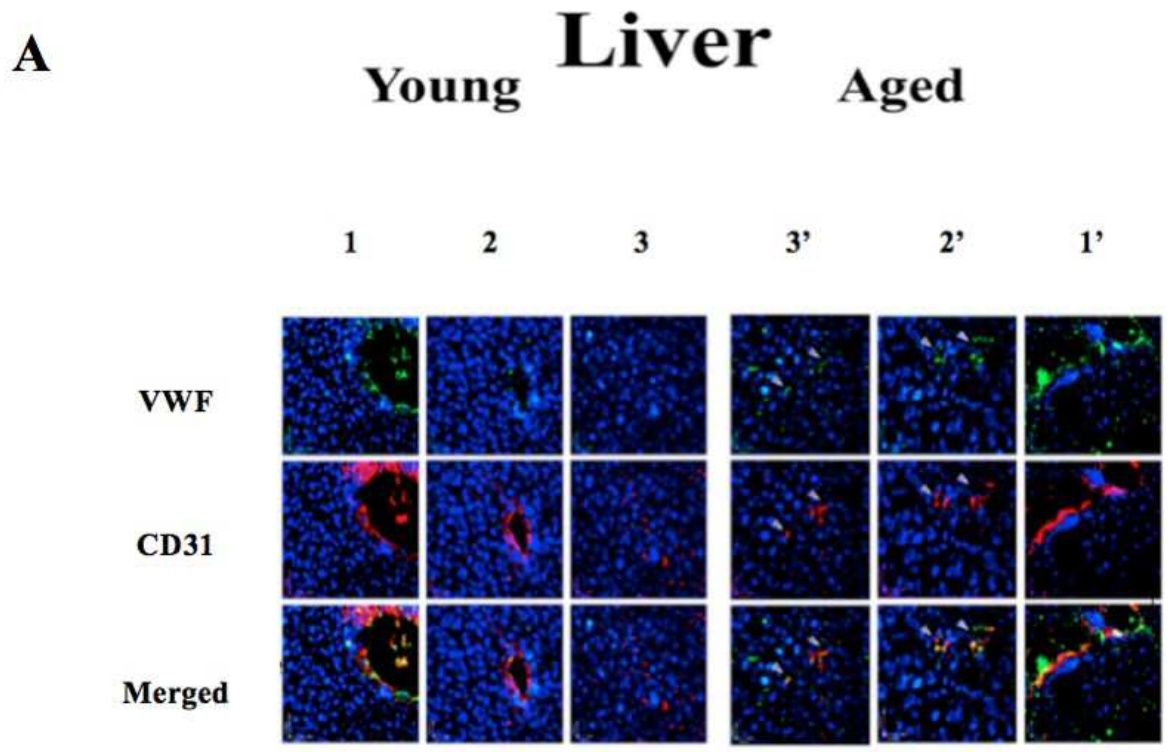
With aging, the increased VWF at mRNA and protein levels were observed in an organ specific manner as shown by RT-PCR and Western blot analysis. However, those analyses did not display the vascular distribution patterns of VWF. To explore that, we proceeded to perform immunofluorescence analyses to investigate the pattern of VWF expression with regard to vascular beds (i.e, vessels size) in organs that exhibit age-related increase in VWF expression. It is well-known that generally VWF is primarily expressed in large vessels in various organs, with microvessels and capillaries exhibiting patchy or no demonstrable VWF protein detection . However, our lab previously demonstrated that in response to hypoxia, there was an alteration in the VWF expression patterns from primarily large vessels to micro vessels in lungs of mice [81]. Therefore, we were interested to determined weather similar changes in the VWF expression patterns might occur with aging. For these analyses, we performed immunofluorescence and confocal microscopy analyses to detect VWF and its colocalization with CD31 or Isolectin GS-IB4. CD31 is a marker of macro and microvascular endothelial cells, while isolectin GS-IB4 specially marks endothelial cells in small vessels in. The immunofluorescence analyses were performed on sections of all major organs (livers, lungs, brains, hearts, and kidneys) of young and aged mice. OCT frozen sections (5 μm) of various organs were processed for immunofluorescence analyses. In these analyses sections of various organs from three young and three aged mice were placed on a single slide. Therefore, multiple tissue

samples were processed simultaneously under similar conditions, thus minimizing inter-experimental staining variability.

4.1 VWF expression pattern in livers of young and aged mice

In livers, the immunofluorescence analyses of VWF expression patterns and its colocalization with CD31 demonstrated that whereas in both young and aged mice VWF was expressed in large and medium sized vessels, a significant number of small vessels only in aged mice exhibited VWF expression (Figure 8A). Significant levels of VWF were detected in large vessels of both young and aged mice (Figure 8A compare panels 1 and 1'). Although medium sized vessels exhibited VWF in both young and aged mice, there appeared to be a significantly lower levels of VWF in these vessel size of young compared to aged mice livers [Figure 8 A compare panels 2 and 2')]. The most significant difference between aged and young mice livers, however, was the complete lack of detectable VWF in small vessels of young while significant number of small vessels in aged mice exhibited clearly detectable levels of VWF. Thus with aging VWF expression patterns were redistributed to include a significant number of microvasculature, while there were no detectable VWF expression by immunofluorescence analyses in microvasculature of young livers [Figure 8 A compare panels 3 and 3']. White arrows indicate the colocalization of VWF with CD31. Detection of VWF in microvascular beds of aged, but not young mice livers was confirmed by performing confocal immunofluorescence analyses for VWF and isolectin GS-IB4 binding, which is a specific marker for microvasculature endothelial cells (Figure 8.B) . White arrows indicate small caliber vessels that exhibit VWF expression and isolectin binding.

The result of immunofluorescence analyses demonstrated that in the aged liver, not only there were increase in the VWF mRNA and protein levels, but the pattern of expression was significantly altered from primarily large vessels in young mice to small vessels (as well as large and medium vessels) in aged mice. There also appeared to be increased levels of VWF in the medium size vessels in aged compared to young mice livers.



Figures 8. A: Representative immunofluorescence staining of Von Willebrand factor (VWF) and CD31 (endothelial cell marker) in livers of young (3 months) and aged (18 months) mice. Five µm OCT frozen sections of mice livers (3 of each young and 3 aged) were placed on one slide. Sections were treated with mouse VWF specific antibody (1:200) raised in sheep, and mouse CD31 specific antibody (1:50) raised in rabbit simultaneously as primary antibodies. Then the sections were treated with the appropriate secondary antibodies donkey anti-sheep Alexa flour 488 (1:1000), and donkey anti-rabbit Alexa 568 (1:1000). Cell nuclei were stained with DAPI followed by confocal microscopy analyses. VWF expression was detected by green, CD31 by red, and colocalization by yellow fluorescent signals (magnification 200X). Results are representative of 3 independent experiments from 6 different mice for each young and aged. Sections of various livers from three young and three aged mice were placed on a single slide, and processed simultaneously under

similar conditions, to minimize inter-experimental staining variability.

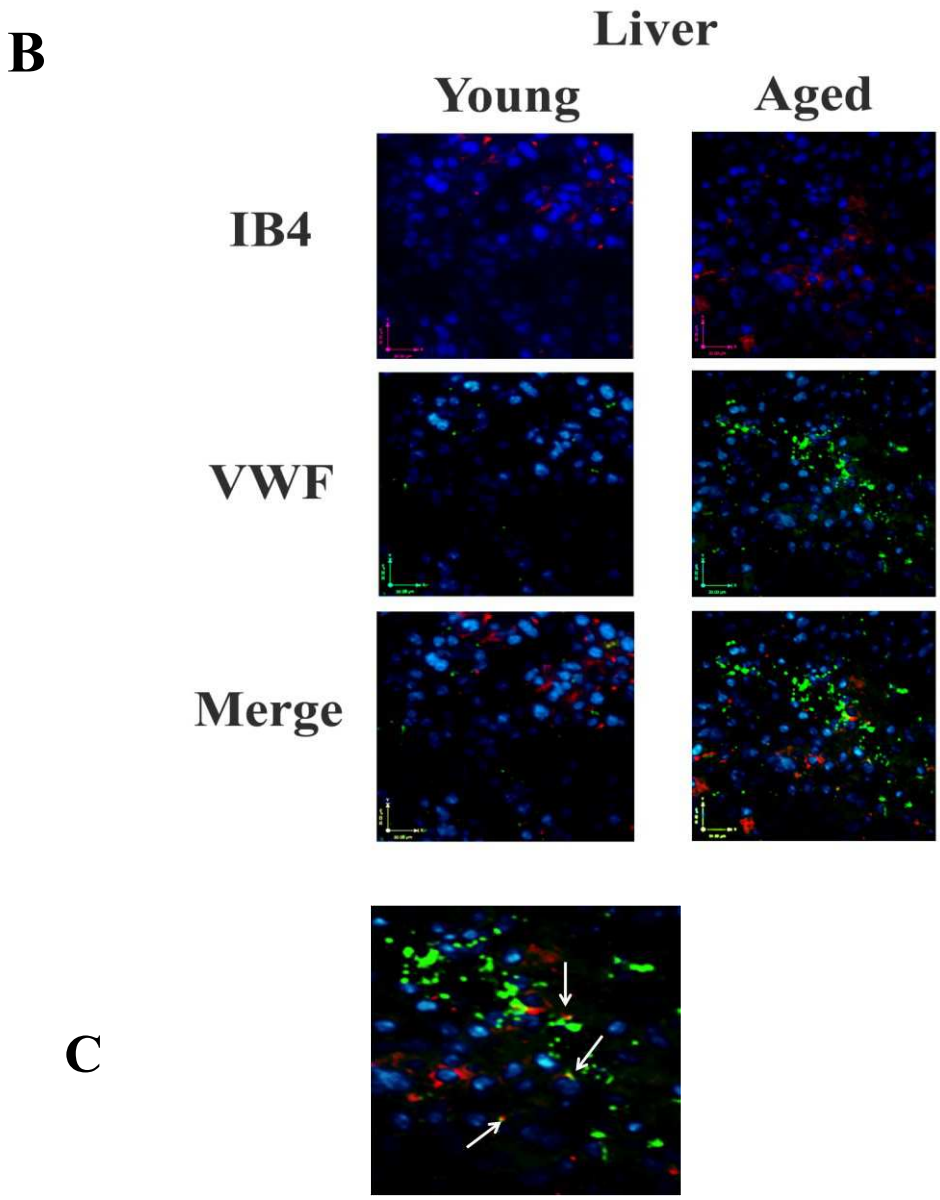
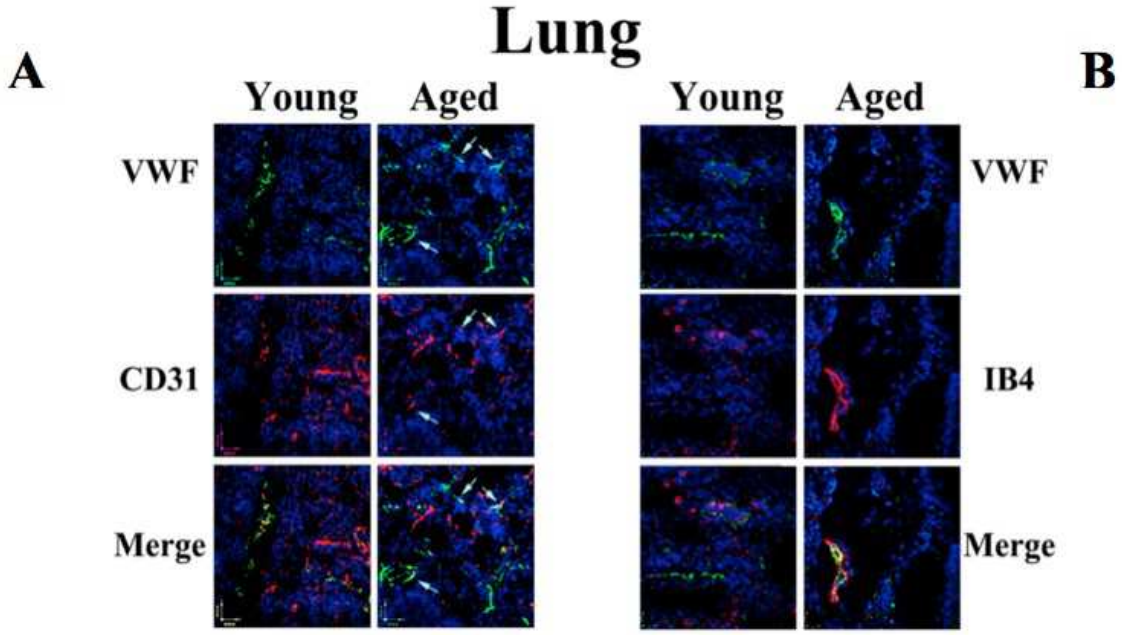


Figure 8B. Analyses of VWF expression and its colocalization to Isolectin GS-IB4-binding endothelial cells of small vessels in livers of young and aged mice. Immunofluorescence and confocal microscopy analyses of VWF and isolectin GS-IB4 were performed on 5-µm OCT frozen sections of liver from young and aged mice as previously described in (figure 8.A). Sections were double stained with Alexa Fluor® 568 conjugated isolectin GS-IB4 (1:200), and the anti-VWF antibody (1:200) as described above for Figure 8A. Cell nuclei were stained with DAPI followed by confocal microscopy analyses. Isolectin GS-IB4 binding was detected as red signal and is labelled as IB4. VWF expression was detected as green signal. Merge shows colocalization of VWF and isolectin GS-IB4, which is detected as yellow signal (Magnification 200X). Results are

representative of 2 independent experiments from 6 different mice for each young and aged. **C)** Magnified section with white arrows showing the VWF colocalization with isolectin GS-IB4 in small vessels.

4.2 VWF expression pattern in lungs of young and aged mice

In lungs, the immunofluorescence analyses of VWF expression patterns and its colocalization with CD31 in aged mice compared to young demonstrated that whereas in young mice VWF was primarily expressed in larger vessels, in aged lungs, VWF expression patterns were redistributed significantly to include a significant number of microvasculature, in addition to larger vessels. There were no detectable VWF by immunofluorescence analyses in lung microvasculature of young mice (Figure 9A). The arrows point to the sites of VWF colocalization with CD31 in small and medium sized vessels in aged lung. Immunofluorescence co-staining analyses using isolectin GS-IB4 and VWF confirmed VWF expression in lung microvasculature of aged mice (Figure 9.B). These results demonstrated that in the lungs of aged mice, not only there were increase in the VWF mRNA and protein levels, but also the pattern of expression was significantly altered. While VWF is expressed mainly in large vessels of young mice it was detected in a significant numbers of small vessels of aged mice. Also there appeared to be increased levels of VWF in the medium sized vessels in lungs of aged mice compared to young (Figure 9B).



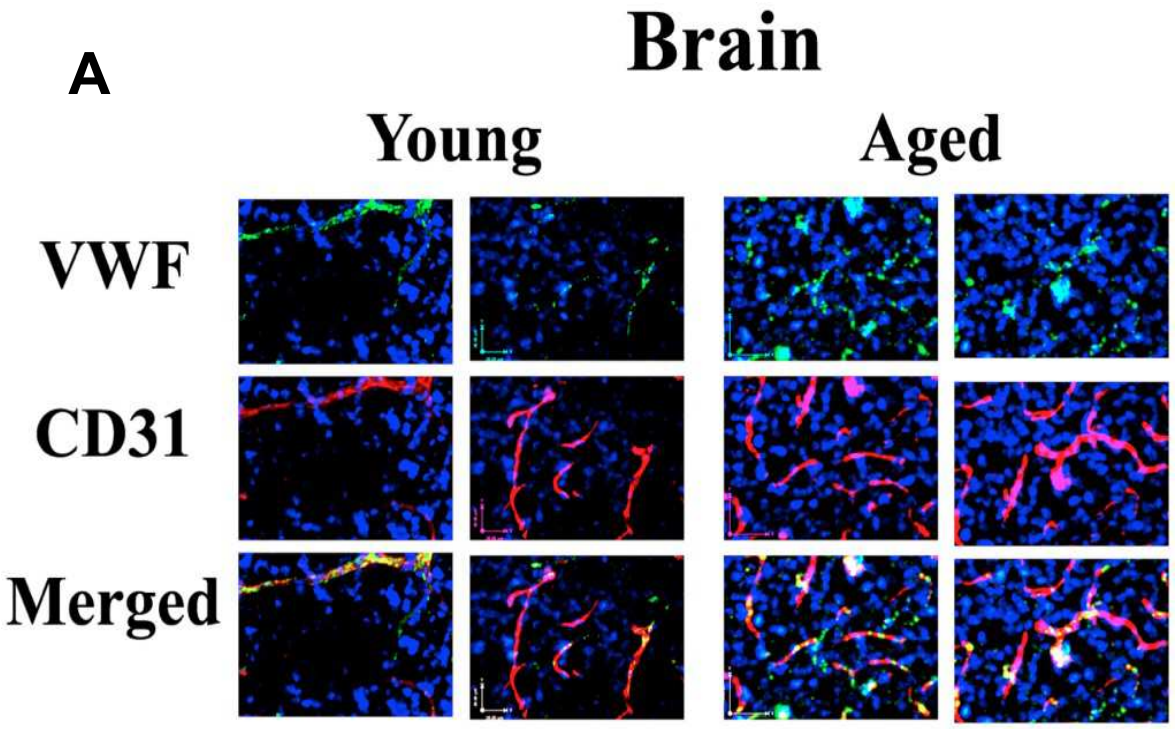
Figures 9 (A) Representative immunofluorescence analyses of VWF and CD31 expression in lungs of young and aged mice (the analyses were performed as described in figure 8 A). Results are representative of 3 independent experiments from 6 different mice for each young and aged.

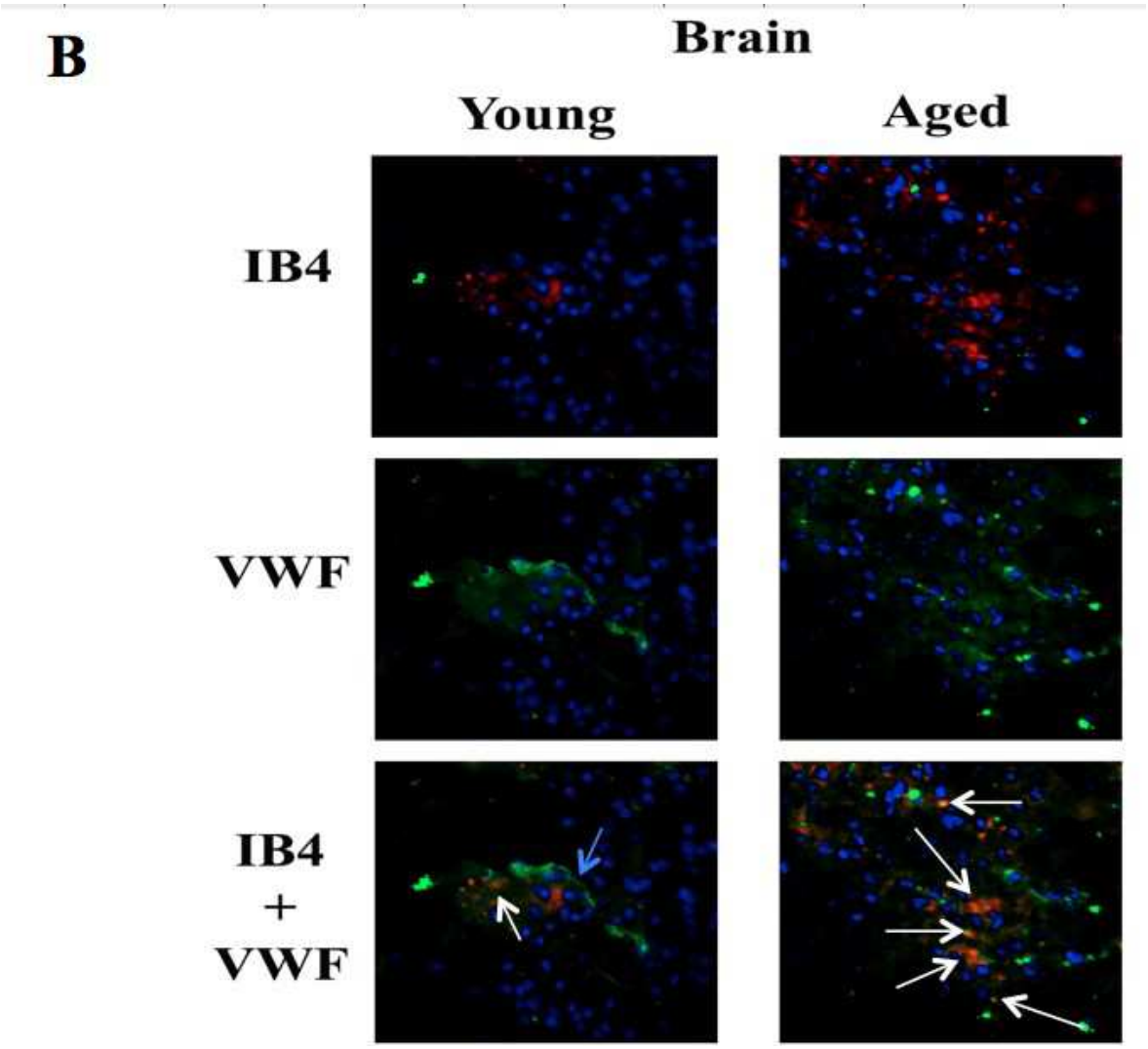
(B) Representative immunofluorescence analyses of VWF expression and isolectin GS-IB4 binding in lungs of young and aged mice. Results are representative of 2 independent experiments from 6 different mice for each young and aged.

4.3 VWF expression pattern in brains of young and aged mice

The immunofluorescence analyses of VWF expression patterns and its colocalization with CD31 in brains of aged mice compared with young demonstrated that in larger vessels of brains, VWF was significantly expressed in both young and aged mice. However, the pattern of VWF expression in microvessels of the brain in young and aged mice were not similar. In brains of young mice, few rare microvessels exhibited low levels of VWF expression. However, significant VWF expression in numerous microvessels of aged mice brains were detected (Figure 10 A). Similar to analyses performed for livers and lungs described above, the expression of VWF in microvessels of the brain were confirmed by costaining with isolectin GS-IB4 and confocal microscopy (Figure 10 B).

These results demonstrated that in the aged mice brains, not only there were increase in the VWF mRNA levels compared to young, but the pattern of VWF protein expression (and most likely the levels of VWF protein) were significantly altered from being primarily detected in large vessels and very few small vessels in young mice, to numerous small vessels, as well as large vessels, in the brains of aged mice.

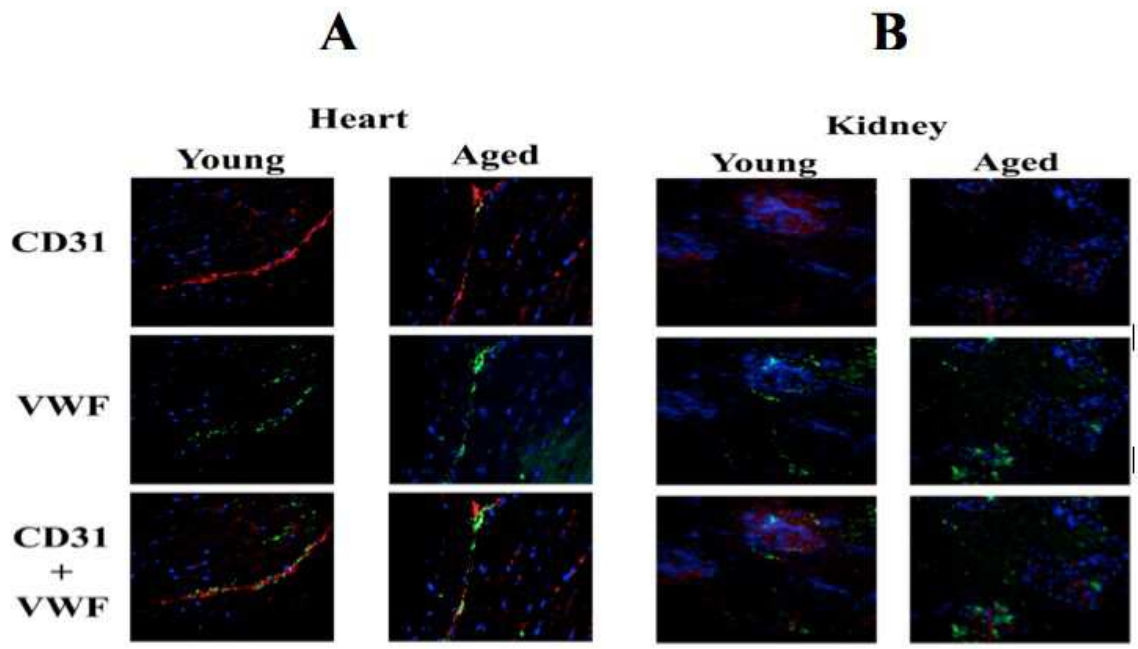

A
Brain
Young
Aged
VWF
CD31
Merged



Figures 10: (**A**)Representative immunofluorescence analyses of VWF and CD31 in brains of young and aged mice (the analyses were performed as described in figure 8 **A**). Results are representative of 3 independent experiments from 6 different mice for each young and aged. **(B)** Representative immunofluorescence analyses of VWF expression and isolectin GS-IB4 binding in brains of young and aged mice. (The analyses were performed as described in figure 8. **B).** White arrows represent VWF colocalization with isolectin GS-IB4 in small vessels. Blue arrow represents VWF expression in a large vessel. Results are representative of 2 independent experiments from 6 different mice for each young and aged. **C)** Magnified section showing the VWF colocalization with isolectin GS-IB4 in small vessels.

4.4 VWF expression pattern in hearts and kidneys of young and aged mice

In contrast to what we have shown in livers, lungs, and brains, our immunofluorescence analyses of the VWF expression patterns in the hearts and kidneys of young and aged mice did not demonstrate a significant alteration in its pattern of expression. VWF expression in these organs of both young and aged mice were restricted primarily to larger vessels (Figure 11). This was consistent with the results of RNA analyses described above that showed a lack of significant change in VWF mRNA levels with aging in hearts and kidneys of mice.



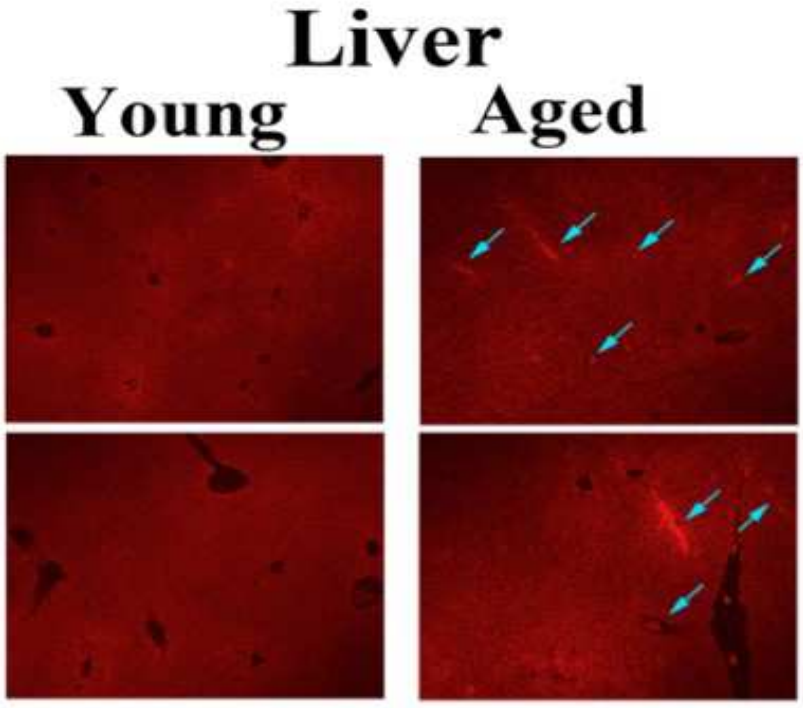
Figures 11. (A) Representative immunofluorescence analyses of VWF and CD31 in hearts of young and aged mice (the analyses were performed as described in figure 8 A). Results are representative of 3 independent experiments from 6 different mice for each young and aged. **(B)** Representative immunofluorescence analyses of VWF and CD31 expression in kidneys of young and aged mice (the analyses were performed as described in figure 8 A). Results are representative of 2 independent experiments from 6 different mice for each young and aged.

5. Platelet aggregate formation in various organs of young and aged mice.

Platelet aggregates formation in livers, lungs and brains of aged mice, is associated with the alteration of VEF expression patterns.

Since VWF is a procoagulant protein that functions primarily to mediate platelets activation and recruitment to endothelial/ subendothelial regions, the observations that with aging-VWF expression was increased in microvasculatures of select organs raised the possibility that this process may contribute to increased platelet aggregations, leading to occlusion of vessels' lumens, which are functional consequences with regard to thrombus formation. To address this question, we analyzed sections of various organs of young and aged mice for the presence of activated platelets by immunofluorescence analyses using CD41, which is a marker of activated platelets. Immunofluorescence analyses of CD41 were performed on 5 μm paraffin embedded sections of various organs. Sections were transferred to slides and processed for immunofluorescence analyses as described in methods. The immunofluorescence analyses demonstrated that increased VWF levels were associated with increased platelet aggregate formation, and detection of occluded vessel lumens in liver, lung and brain. (Figures 12,13,&14). Consistent with the lack of age related alterations in VWF expression in hearts and kidneys, significant increase in platelet aggregate formation and vessel occlusions were not detected in these organs (Figures 15 and 16). We quantified the occluded vessels (those exhibiting 50-80-% occlusion) in all major organs stained with CD41. The analyses demonstrated that in livers and lungs of aged mice, the number of occluded vessels increased three-fold in comparison to young mice,

and the platelet aggregates were detected in medium and small sized vessel; whereas in brains of aged mice the number of occluded vessels increased seven-fold compared to young, and the platelet aggregates were detected in a significant number of small sized vessels. These results collectively demonstrate that increased VWF levels and redistribution pattern develops with aging in an organ-specific manner, specifically in lung, liver and brain, and this process correlates with increased formation of activated platelets aggregate and vessel occlusion.



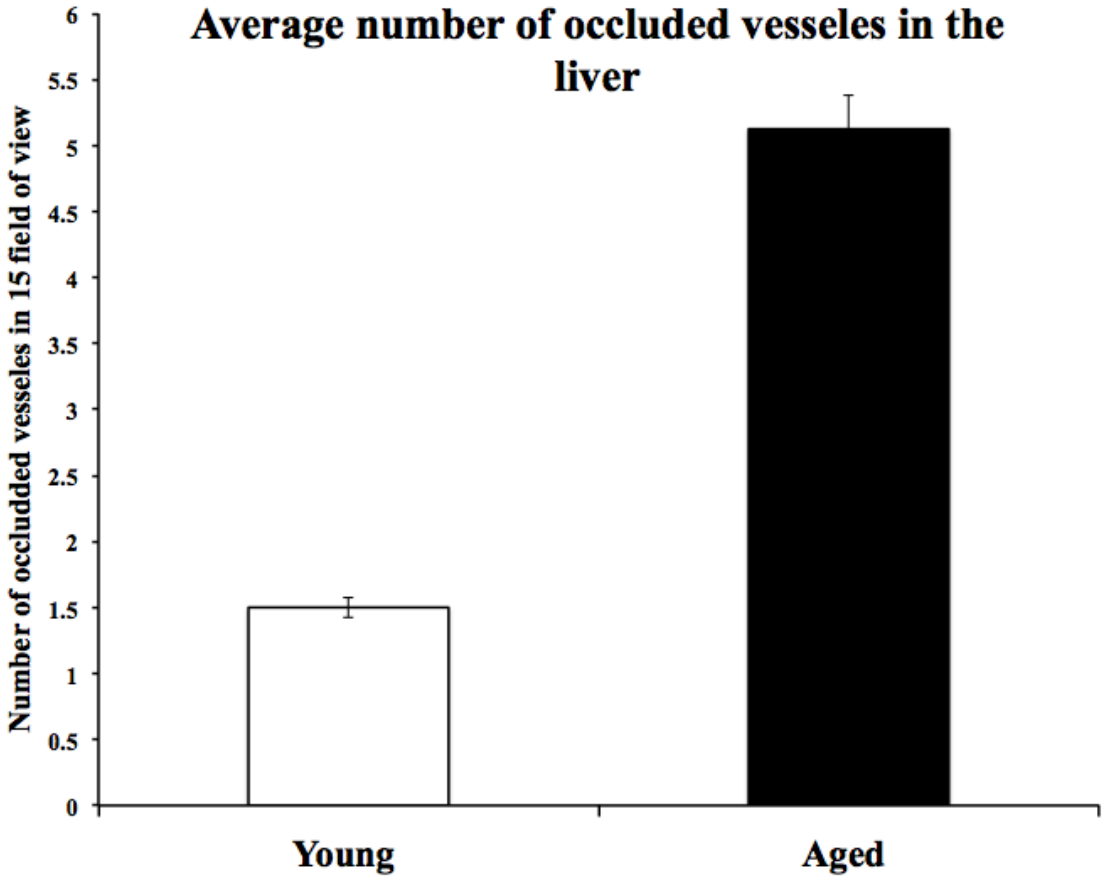
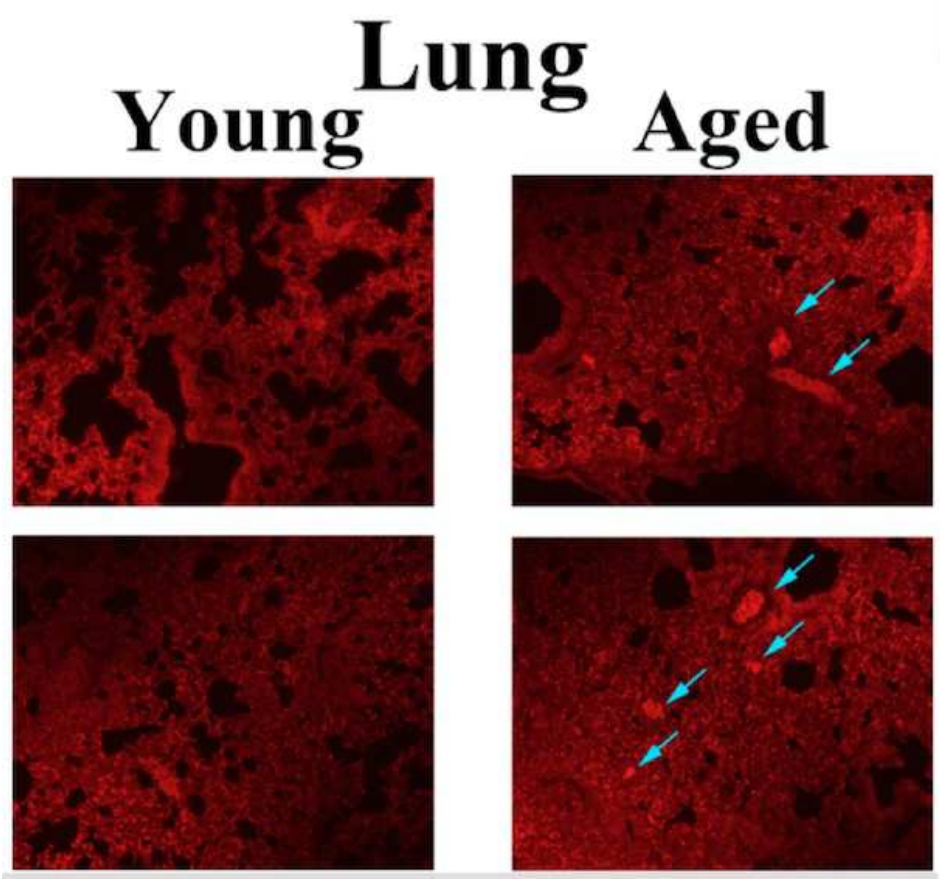


Figure 12 A. Representative immunofluorescence staining for CD41 (a marker of activated platelets) in livers of young and aged mice. 5 μm paraffin embedded sections of liver (3 each young and aged) were placed on one slide. Sections were treated with mouse CD41 specific antibody (1:50) as a primary antibody, followed by treatment with secondary antibody [(1:1000) donkey anti-rat Alexa 568]. Cell nuclei were stained with DAPI. The platelet aggregates formation (red) are shown by arrows. Results are representative of 2 independent experiments from 6 different mice for each young and aged. Vessels that contained platelet aggregates which occluded the lumen 50-80% were quantified in 15 field of views for each independent staining experiment and averages presented in the graph (* $P<0.05$).


Lung
Young
Aged

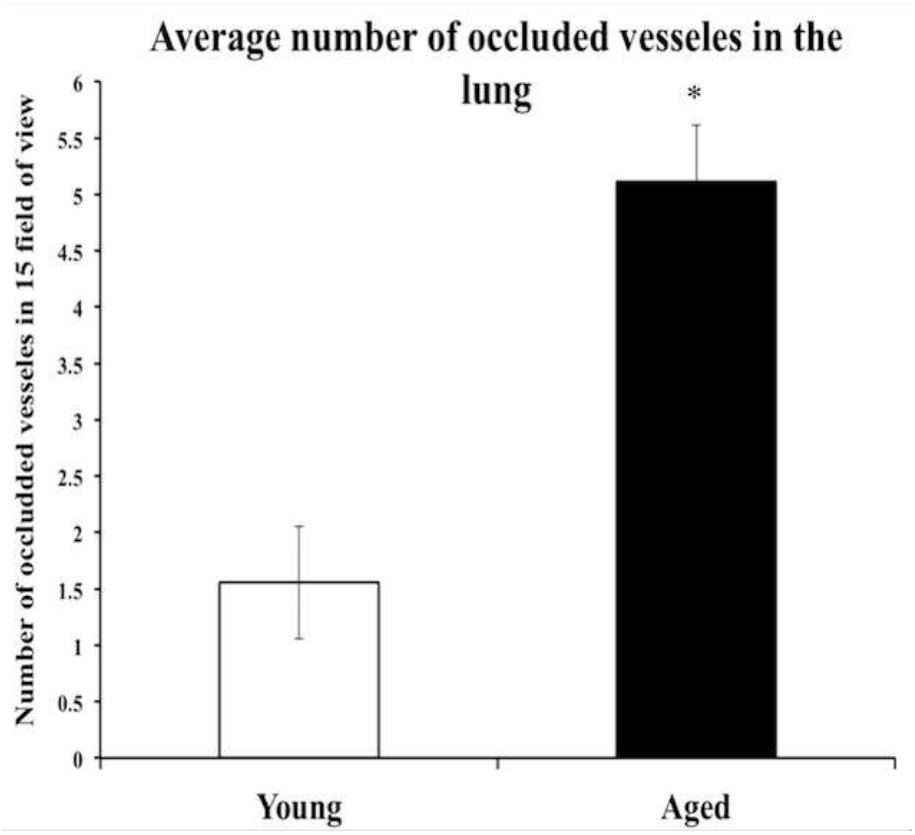
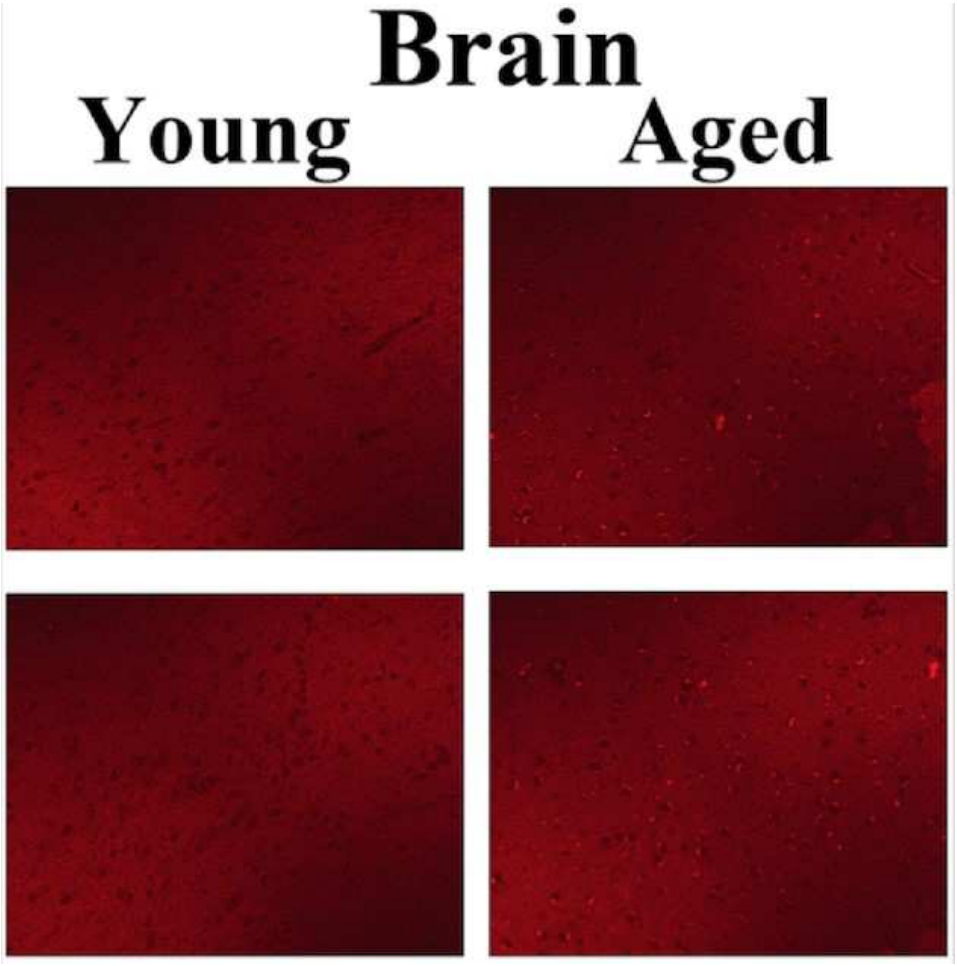

Average number of occluded vesseles in the lung
Number of occludded vesseles in 15 field of view
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Young
Aged

Figure 13. Representative immunofluorescence staining for CD41 (a marker of activated platelets) in lungs of young and aged mice. 5 μm paraffin embedded sections of lung (3 each young and aged) were placed on one slide. Sections were treated with mouse CD41 specific antibody (1:50) as a primary antibody, followed by treatment with secondary antibody [(1:1000) donkey anti-rat Alexa 568]. Cell nuclei were stained with DAPI. The platelet aggregates formation (red) are shown by arrows. Results are representative of 2 independent experiments from 6 different mice for each young and aged. Vessels that contained platelet aggregates, which occluded the lumen 50-80% were quantified in 15 field of views for each independent staining experiment and averages presented in the graph (* $P<0.05$).



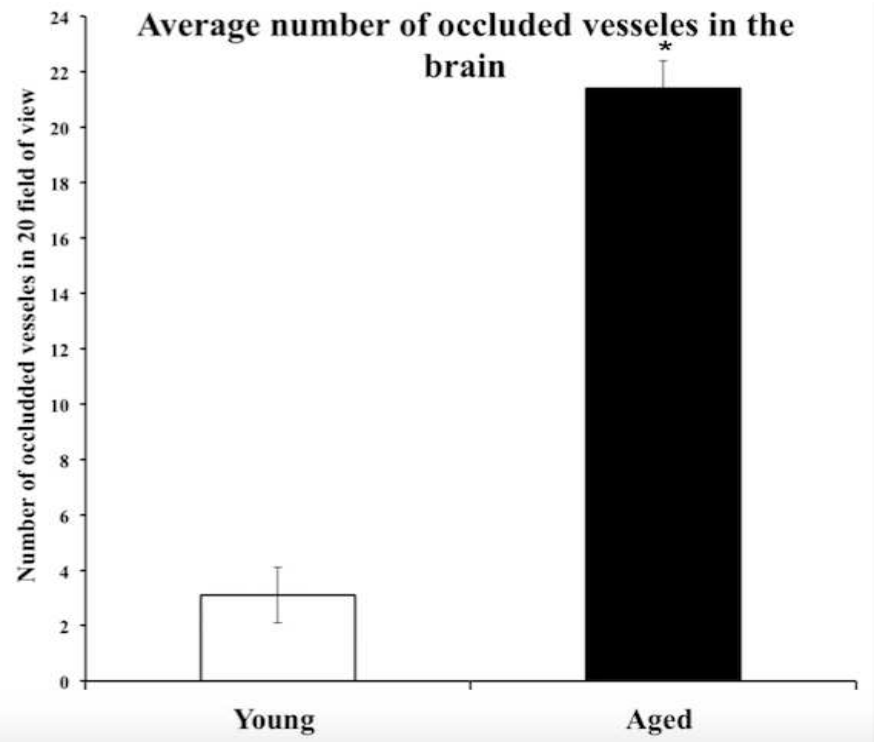


Figure 14. Representative immunofluorescence staining for CD41 (a marker of activated platelets) in brains of young and aged mice. 5 μm paraffin embedded sections of brain (3 each young and aged) were placed on one slide. Sections were treated with mouse CD41 specific antibody (1:50) as a primary antibody, followed by treatment with secondary antibody [(1:1000) donkey anti-rat Alexa 568]. Cell nuclei were stained with DAPI. The platelet aggregates formation (red) are shown by arrows. Results are representative of 2 independent experiments from 6 different mice for each young and aged. Vessels that contained platelet aggregates, which occluded the lumen 50-80% were quantified in 15 field of views for each independent staining experiment and averages presented in the graph (* $P<0.05$).

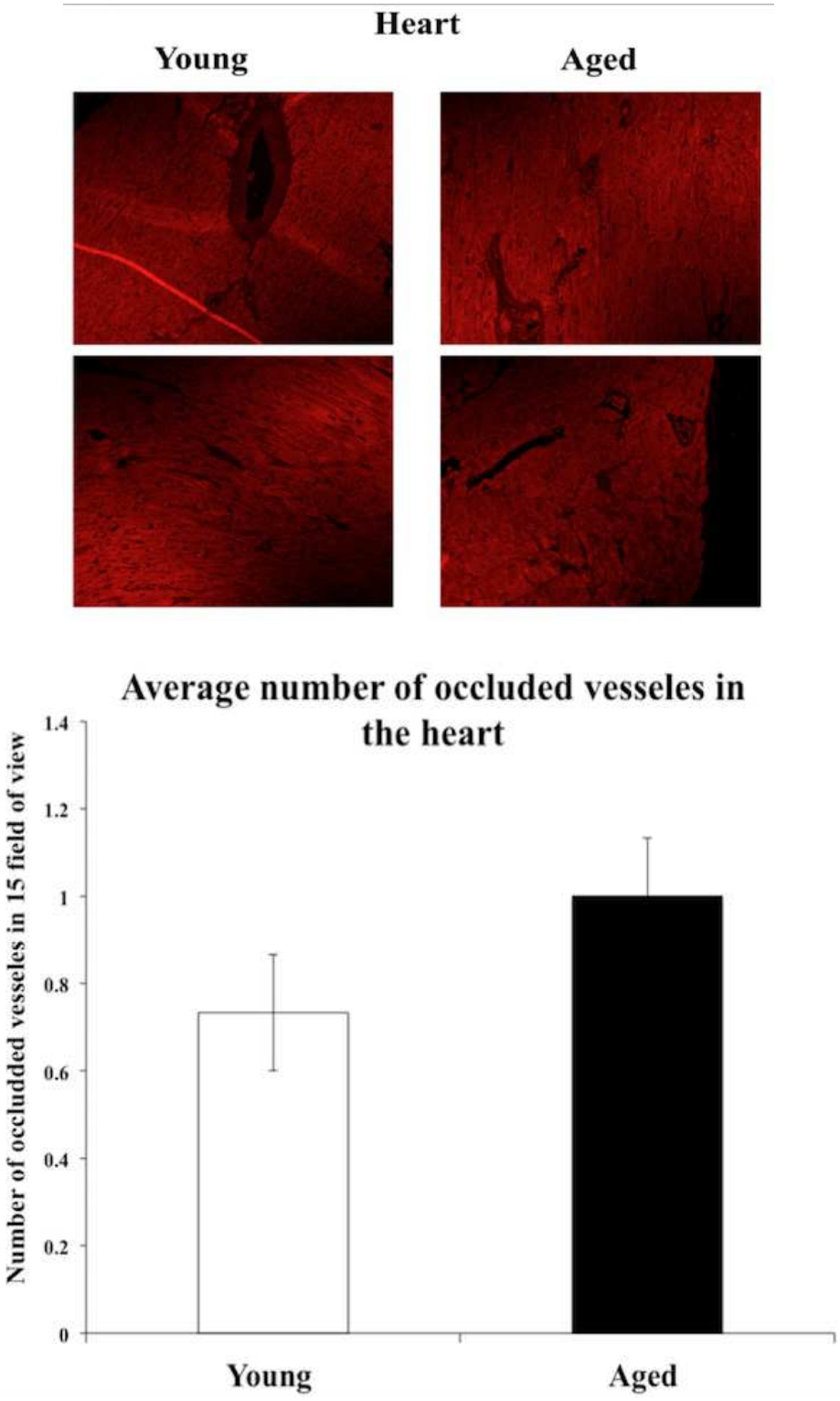
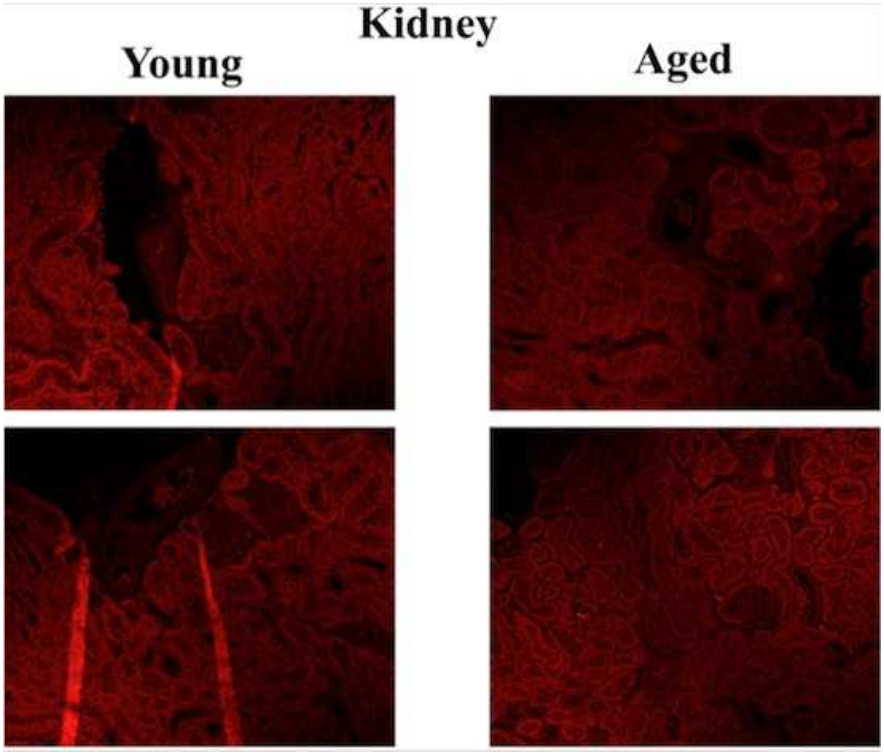


Figure 15. Representative immunofluorescence staining for CD41 (a marker of activated platelets) in hearts of young and aged mice. 5 μm paraffin embedded sections of heart (3 each young and aged) were placed on one slide. Sections were treated with mouse CD41 specific antibody (1:50) as a primary antibody, followed by treatment with secondary antibody [(1:1000) donkey anti-rat

Alexa 568]. Cell nuclei were stained with DAPI. The platelet aggregates formation (red) are shown by arrows. Results are representative of 2 independent experiments from 6 different mice for each young and aged. Vessels that contained platelet aggregates, which occluded the lumen 50-80% were quantified in 15 field of views for each independent staining experiment and averages presented in the graph (* $P<0.05$).



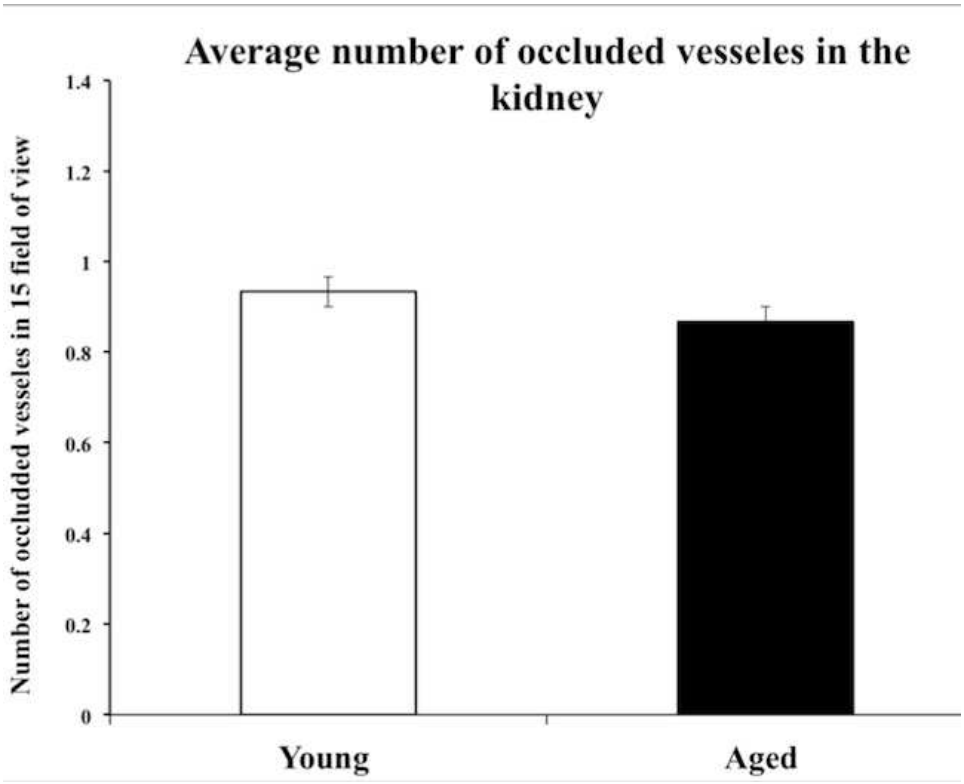


Figure 16. Representative immunofluorescence staining for CD41 (a marker of activated platelets) in kidneys of young and aged mice. 5 μm paraffin embedded sections of kidney (3 each young and aged) were placed on one slide. Sections were treated with mouse CD41 specific antibody (1:50) as a primary antibody, followed by treatment with secondary antibody [(1:1000) donkey anti-rat Alexa 568]. Cell nuclei were stained with DAPI. The platelet aggregates formation (red) are shown by arrows. Results are representative of 2 independent experiments from 6 different mice for each young and aged. Vessels that contained platelet aggregates, which occluded the lumen 50-80% were quantified in 15 field of views for each independent staining experiment and averages presented in the graph (* $P<0.05$).

CHAPTER 4: Discussion

There are many pathological, physiological, environmental, and genetic factors that alter the levels of VWF in circulation and alter VWF expression patterns in tissues either by decreasing or increasing its levels. Decreased VWF levels cause VWD, while increased VWF levels contributes to increased thrombogenicity. High VWF levels are associated with an increased risk for cardiovascular disease [73,51]. Age is considered a risk factor for cardiovascular disease, and the elevated plasma levels of VWF in aged population have been documented in some studies [13]. These studies have reported increased circulating levels of VWF in aged compared to young adults and an associated increased risk of venous thromboembolism and cardiovascular diseases (CVD) [13].

Since previous studies were focused on determining the alterations in circulating VWF levels with aging, we aimed to study whether process of aging may contribute to changes in VWF expression at RNA and cellular protein levels, which could reflect transcriptional activity. We also aimed to determine whether vascular bed distribution pattern of VWF is altered with aging which may reflect phenotypic alterations of endothelial cells with regard to pro and anti-coagulant activities.

VWF levels in aged human

There are many studies in which VWF levels were measured in pediatric and aged population, and they have documented that VWF plasma levels are increased in healthy aged people in comparison to young adults [45]. David Lillicrap and colleagues [46] studied the effect of aging and ABO blood type on plasma levels of VWF and FVIII, and

showed that the age-related increase in plasma levels of VWF and FVIII was significantly higher in type non-O individuals, and they found that the presence of both aging and A/B antigens had a major effect on VWF plasma levels, in contrast to when only one factor was present [46]. They examined the mechanisms that contribute to the age-related changes by evaluating specific markers such as detecting VWF secretion and clearance in normal young and aged population. The study was performed with a total of 207 normal participants who were categorized into three age groups: (52 young, mean age 17 years), (42 middle age, mean age 41 years), and (113 old, mean age 71 years). To minimize the interchanges, the three age groups were similar in gender and ABO blood type composition. They found that plasma levels of VWF increased from a mean value of 0.13 U/mL in young individuals aged 17 years to 0.57 U/mL in the mean age group of 71 years. They showed that the age-related increase in plasma levels of VWF and FVIII was significantly higher in type non-O individuals [46].

In this study FVIII levels were also shown to be influenced by aging and ABO blood type. However, the changes in FVIII levels were dependent on the changes in VWF. FVIII circulates in a complex with VWF, which stabilize and protects it from early degradation, thus usually any elevation in VWF levels contributes to an increase in FVIII levels [46, 39, 35]. In the young population, levels of VWF and FVIII were not significantly different. However, in aged population, the levels of VWF and FVIII increased with aging. This age associated increase was blood type dependent and most significant in individuals with blood type non-O [46].

The study also explored VWF clearance in relation to aging. By measuring the VWF propeptide to VWF: Antigen ratio (VWFpp)/ (VWF: Ag) distinct changes in VWF

clearance with aging and blood types were demonstrated . The differences in (VWFpp)/ VWF: Ag) were observed only in the advanced age poulation, but not in the young population. Additionally, no significant differences in VWFpp levels throughout aging were observed in individuals with blood type O, whereas VWFpp levels were significantly elevated in old individuals with blood type non-O. They also documented the effect of ABO blood type on VWF secretion with aging, as old individuals with blood type non-O showed higher levels of VWF propeptide (VWFpp) with mean difference value of 0.29 U/mL [46].

Nevertheless, none of these studies considered the proposal that with aging, the transcriptional activity of the VWF gene, potentially reflected in RNA levels and patterns of VWF expression may be altered in different organs and different types of vascular beds.

VWF levels and expression patterns in aged mice.

In our study, we found that plasma levels of VWF are increased in aged rats and mice. This finding was supported by other previously mentioned studies in aged human population, and based on this observation, we pursued to explore whether this was reflected in alteration in VWF transcriptional levels. Accordingly, we proceeded to determine whether VWF mRNA levels were different in various organs of aged compared to young mice. We also proceeded to investigate whether VWF displayed heterogeneity regarding its expression pattern in different vascular beds of various organs.

First, circulating VWF levels were determined for young and aged rats and mice at various ages as follows: young rats (3 months, which are similar to human age of 15 years)

compared to aged rats (19 months, which are similar to human age of 45 years); young mice (3 months, which are similar to human age of 23 years) compared to aged mice (18 months, which are similar to human age of 65 years) . The results showing eleveated levels of plasma VWF in aged rats and mice were consistent with the elevated plasma VWF levels in aged human populations [46]. Additionally, the results demonstrated that the phenomenon may be more pronounced in some species like rats since aged rats used in our study had a human age equivalent of 45 years. Alternatively the results may suggest that further studies in human population, using a larger cohort of participants, may reveal significant differences at less of an age range.

Next, we looked at VWF mRNA, and protein levels in all major organs (livers, brains, lungs, hearts and, kidneys) of young and aged mice. There was a significant increase in the mRNA levels of VWF in livers, brains, and lungs, but not hearts, and kidneys of aged mice compared to young. This strongly indicates that age related VWF transcriptional activities are different in various organs, and thus VWF displays organ-dependent heterogeneity in its transcriptional response to aging. Results of Western blot analysis were consistent with those of mRNA analyses and showed increase in VWF cellular protein levels in the same organs (liver, brain and lung) of aged mice compared to young. The results of these analyses also demonstrated that there were significant differences in the levels of VWF mRNA in various organs of similar aged mice, which was consistent with previous reports (31). This potentially represents the difference in the levels of vascularization of various organs, and differential VWF expression in different vascular beds. Regardless of well – established differences in the levels of VWF mRNA among various organs our findings demonstrated that with aging only select organs namely, lung, liver, and brain exhbit

increased VWF mRNA levels. This observation suggests that transcriptional activity of the VWF promoter in livers, brains, and lungs may be upregulated with aging. Although, in this study we did not explore the roles of transcription factors that may be involved in age-induced transcriptional upregulation of the VWF promoter, the significance of our finding is the organ-specific elevation in VWF mRNA and cellular protein levels with aging which suggests a mechanism that could point to alterations in transcriptional activities of endothelial cells of distinct organs.

Generally, there is also difference in VWF expression among different parts of vascular tree in each organ. For instance, normally in kidneys glomeruli don't express VWF, also the sinusoidal endothelial cells in liver don't express VWF; whereas, the large vessels in both kidney and liver significantly express VWF. In lung also the VWF expression is not uniform, it is primarily detected in larger vessels [66, 31], with expression ranging from patchy to undetectable as the vessel size decreases from medium sized vessels to capillaries. However, it has been shown that the patterns of the endogenous VWF expression are significantly altered in response to some stimuli. For example, studies by our group demonstrated that there was a significant alteration in the lung VWF expression patterns in response to hypoxia from primarily large vessels in control mice to micro vessels in hypoxic mice [31]. Therefore, we analyzed the patterns of VWF expression to determine whether this type of changes in the VWF expression patterns could also occur with aging.

The results of immunofluorescence analyses of VWF expression (using endothelial cell markers for both large vessels and microvasculature) in various organs of aged and young mice demonstrated that in livers, brains and lungs, but not hearts and kidnys, VWF

expression pattern is altered with aging. This organ-specific alteration in expression pattern was consistent with Western blot and RNA analyses that did not demonstrate a significant change in the VWF mRNA and protein levels in heart and kidneys with aging.

In the aged livers and lungs, not only there were increase in the VWF mRNA and protein levels, but also the pattern of expression was significantly altered from primarily large vessels to include microvessels. Also increased VWF levels were indicated in the medium sized vessels in livers and lungs of aged mice. Similarly, in the brains of aged mice, not only there were increase in the VWF mRNA levels, but also the pattern of expression was significantly altered from primarily large vessels (and few small vessels) in young mice to a significant increase in the number of small vessels that expressed VWF (in addition to the large vessels). Based on these results, we concluded that microvasculatures of young liver lung, and brain do not express any (or very little in case of brain) detectable VWF, the patterns of VWF expression were altered significantly with aging and microvasculature of all three organs exhibited VWF expression. The lack of this alteration in VWF expression pattern in heart and kidney underscores the heterogeneity in vascular endothelial cell phenotype of various organs.

We studied the functional consequences of elevated and altered pattern of VWF expression with aging. We were interested to investigate whether there is a correlation between increased levels of VWF and the activated platelet aggregations, which may cause occlusion of the vessels lumens. This is a potential functional consequence of elevated VWF levels that could result in thrombus formation.

The results of immunofluorescence analyses to detect activated platelet aggregates in

various organs demonstrated that with aging, significant platelet aggregates formations were detected in the livers, brains, and lungs (but not heart and kidneys), which lead to 50-80% occlusion of numerous small sized and some medium sized vessels. Thus functional consequences for increased/altered VWF expression with regard to platelet aggregate formation, leading to vessel occlusion, were demonstrated and were consistent with organ-specific alteration in VWF expression; i.e functional consequences were demonstrated selectively in organs (lung, liver and brain) showing increased VWF, but not in heart and kidney that did not exhibit age related alteration in VWF expression. The results demonstrated that change of VWF expressions reflect an increased platelet aggregation activity.

Diseases associated with aging

As we have observed in this study, with aging there were increases in plasma, cellular protein, and mRNA levels of VWF; as well as alteration in the vascular patterns of VWF expression in livers, lungs and brains. These changes are expected to have important clinical implications with regard to diseases in these specific organs. Increase VWF expression in these three major organs may contribute to decline in their blood flow rates. It has been reported that a significant percentage of morbidity and mortality in the elderly is attributed to liver disease [84,85]. It also has been documented that the number of patients older than 65 years of age with chronic liver disease has increased, and the number of elderly with age-related liver diseases will increase markedly throughout the next twenty years [84,63].

Brain undergoes definite decline in structure and function with age [37]. Based on our analyses, which demonstrated that in the brains of aged mice the number of occluded vessels increased seven-fold compared to young, it is conceivable that this process may contribute to decreased perfusion of brain tissues, thus a contributing factor to decline in brain function. Additionally this may increase the propensity to thrombogenicity and thus increased risk of stroke. Decrease in the blood flow to the brain is associated with several brain disorders including vascular dementia that occurs when clots block blood flow to parts of the brain, subsequently damaging under-perfused brain tissues. Symptoms of vascular dementia vary widely, depending on the brain regions involved. Common symptoms include memory loss, difficulty focusing attention and confusion [38]. Older people often experience decreased blood flow to the brain, which can impair memory [32].

Since the vascular endothelium plays an important role in the normal hemostasis process, any structural or functional change in the vascular wall, like those that could occur during aging, may contribute to the increased risk of thrombosis in the elderly [83]. There is a higher increase in the risk of arterial and venous thrombosis with aging, which indicates that arterial and venous thromboses are age related diseases [83]. One of the main risk factors that cause the arterial and venous thrombosis is elevated levels in plasma concentrations of some coagulation factors (factors V, VII, VIII, and IX, fibrinogen), which increase significantly with aging [83]. However, VWF was also recently shown to exhibit significantly higher plasma levels in type non-O aged individuals [72] compared to young. The important role of VWF in thrombus formation in arteries and veins has been well documented [77,84,90]. A number of studies have demonstrated the increased plasma levels of VWF in patients with atherothrombotic and its association with high risk of

thrombogenicity [31]. Even though, the hemostatic system is generally shifted towards a more procoagulant state with aging [83], there is a gap in our knowledge regarding the contribution of VWF and the underlying mechanism of its increased circulating levels with aging

There are many age-associated loss of kidney function that has been recognized, including a significant decrease in glomerular filtration rate (GFR), and renal blood flow (RBF) [86]. Many epidemiologic studies have demonstrated that acceleration of age-related loss of renal function might be associated with risk factors such as systemic hypertension, atherosclerotic disease [87], obesity, lead exposure, smoking, acute kidney injury, and presence of inflammatory markers among others [88]. Generally, kidney diseases are not age-related diseases, but due to other risk factors. It has been shown that in rats at the equivalent age of human late middle age, the glomerular capillary plasma flow rate, and single nephron GFR (SNGFR), remained similar to those in young rats [86]. VWF levels and secretion do not change in aged kidney without any other contributing factor. For instance, high secretion of VWF leading to thrombus formation in kidneys of aged mice, leading to renal injury, was observed when mice were lacking endothelial nitric oxide synthase (as a result of gene knock out), but not in wild type mice [60]. These studies are consistent with our results demonstrating a lack of age –associated altered VWF expression in kidneys.

With aging, VWF levels are increased in circulation. Furthermore, an altered VWF expression pattern, specifically increased expression in microvasculature of brain, lung, and liver, is observed. Overexpression of VWF in circulation and microvasculature vessels

of distinct organs as a result of aging may contribute to vascular diseases such as thrombosis.

The findings of this study could provide insights toward future therapeutic anti thrombotic development for aged people.

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