Mechanism and consequences of von Willebrand factor upregulation in response to aging and organ transplantation

by

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Abstract

Von Willebrand factor (VWF) is a procoagulant protein, which is crucial for blood clot formation and is exclusively expressed in endothelial cells and megakaryocytes. An unregulated increase in VWF levels is associated with elevating the incidence of thrombosis. Elevated VWF levels are observed in pathological conditions as well as physiological conditions and in response to various stimuli and could be a major contributing factor in the association of such conditions with increased thrombogenicity.

Aging is the primary risk factor for cardiovascular disease, which is the most prevalent cause of morbidity and mortality in elderly populations. Several studies have reported that aging increases the circulating levels of VWF, leading to shifts in the hemostatic balance toward pro coagulability and prothrombotic state. While it has been widely reported that circulating VWF levels rise in normal-aged individuals compared to young individuals, there is a gap in our knowledge regarding the mechanism by which aging leads to increased circulating levels of VWF. In this study, I have explored the mechanism by which an age-associated increase in VWF occurs and determined its functional consequences with respect to increased thrombogenicity.

Our analysis demonstrated plasma levels of the antigen and high molecular weight multimers form of VWF were increased in aged mice compared to young. Both VWF mRNA and cellular protein were increased in an organ-specific manner. It increased in the brains, lungs, and livers but not in the kidneys and hearts of aged mice compared to young. There was an increase in the number of microvascular endothelial cells that exhibited VWF expression in the vasculature of aged organs (liver, lung and brain) compared to young. Investigation of the functional consequences of increased VWF levels demonstrated that it was concomitant with a significant increase in platelet aggregates formation in the vascular beds of target organs of aged but not young

mice. In vitro analyses demonstrated that there was a significant increase in VWF mRNA levels, in prolonged culture of endothelial cells; and associated with cells that exhibited senescence. Higher proportion of VWF expressing endothelial cells in vivo exhibited senescent markers β-galactosidase and p53 in aged mice brain compared to young. Knockdown of p53 in cultured endothelial cells eliminated VWF upregulation in time-extended/senescent cultured cells, demonstrating the participation of p53 in this process. Moreover, treatment of aged mice with lipid nanoparticles (LPN), which target p53 expressing senescent cells for destruction, exhibited a significant reduction in senescent brain microvascular endothelial cells, and platelets aggregate formation. These results provide insight into designing potential organ-specific targeted treatments to address age-related thrombogenic complications.

Another condition, which may occur physiologically (embryonic development) or pathophysiologically (tumor progression) and is demonstrated to induce VWF upregulation is hypoxia. Our group has reported hypoxia can upregulate VWF and alter its vascular bed expression pattern in lung. Lung transplantation is an effective therapeutic intervention and conventionally necessitates exposure of donor lung to a period of hyperthermia and hypoxia. Since thrombosis is a major complication of lung transplantation, which may lead to allograft failure and since during organ-transplantation donor organs are exposed to hypoxic conditions, we explored whether this may lead to alterations in VWF expression. We also aimed to determine whether an innovative organ preservation method, ex vivo lung perfusion (EVLP) prior to transplantation, could moderate this potential effect and as a result reduce its thrombogenic consequences.

To test our hypothesis, we procured pig's lungs maintained under conventional static cold storage conditions (SCS), and those in EVLP. Tissue biopsies were obtained at the beginning and the end of SCS period or EVLP perfusion for further analysis. The results demonstrated that VWF

mRNA and protein levels in lungs maintained under SCS were unaltered, but significantly reduced in lungs that were perfused under ex vivo lung perfusion “EVLP” for 12 hours. This result was not limited to lungs; similar results were observed in limb’s blood vessels preserved under ex vivo setting. Furthermore, IF analysis of donor lungs post transplantation demonstrated that the preserving lungs in cold storage conditions, but not EVLP, prior to transplantation altered VWF expression pattern, leading to an increased number of microvascular endothelial cells that express VWF in transplanted lungs. These Results provide insights into developing effective anti-thrombotic approaches that would be advantageous in organ transplant procedures.

Preface

This thesis is an original work by Parnian Alavi. Experimental design, performance and analysis of data were completed by Parnian Alavi and her supervisor Dr. Nadia Jahroudi.
This thesis consists of two published works and one unpublished work, which has been prepared for submission to a peer-reviewed journal.

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Dr. John P. Cooke from the Houston Methodist research institute provided the organ sections from wild-type and progeria mice. All animal experiments were performed following the guidelines approved by the Canadian Council for Animal Care (CCAC), and the University of Alberta Animal Care and Use Committee approved the animal protocol.

Dedications

To my family
Where life begins & love never ends

No matter how old you get, your family's love and support will never change

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List of Abbreviations

ADAMTS13	A disintegrin and metalloproteinase with a thrombospondin type 1 motif member
ARDS	Acute respiratory distress syndrome
ASA	Aspirin
BT	Bleeding time
DAPI	4',6-diamidino-2-phenylindole
E4BP4	E4 promoter-binding protein 4
ECs	Endothelial cells
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
Ets	E-twenty-six
EVLP	Ex vivo lung perfusion
FACS	Fluorescence-activated cell sorting
GATA	GATA-binding protein
GPIb	Glycoprotein Ib
HCU	Heater/Cooler Unit
HDAC	Histone deacetylase
HGPS	Hutchinson-Gilford progeria syndrome
HLP	H1-like protein

HMWM	High molecular weight multimers
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
HSS	Hypersensitive
HUVEC	Human umbilical vein endothelial cells
ICU	Intensive care unit
IF	Immunofluorescence
KHB	Krebs-Henseleit buffer
LA	Left atrium
LAP	Left atrial pressure
LMVECs	Lung microvascular endothelial cells
LNP	Lipid nanoparticles
MAC	Membrane attack complex
NAC	N-acetylcysteine
NFAT5	Nuclear factor of activated T cells
NFI	Nuclear factor I
NFY	Nuclear factor Y
NO	Nitric oxide
NPV	Negative pressure ventilation
OCT	Optimal cutting temperature
Oct1	Octamer-binding transcription factor 1
PACE	Paired basic Amino acid Cleaving Enzyme
PAP	Pulmonary artery pressure
PCAF	p300/CBP-associated factor

PCR	Polymerase chain reaction
PE	Pulmonary embolisms
PH	Pulmonary hypertension
PPV	Positive pressure ventilation
pRBC	Packed red blood cells
rADAMTS13	Recombinant ADAMTS13
SA-β-Gal	Senescence-associated β-galactosidase
SCN	Suprachiasmatic nucleus
SCS	Static cold storage
SDS	Sodium dodecyl sulfate
SiRNA	Small interfering RNA
TTP	Thrombotic thrombocytopenic purpura
UL-VWF	Ultra-large von Willebrand factor
VCI	Vascular cognitive impairment
VSMCs	Vascular smooth muscle cells
VWD	von Willebrand disease
VWF	von Willebrand factor
VWF:Ag	von Willebrand factor antigen
VWFpp	von Willebrand factor propeptide
WPBs	Weibel-Palade bodies
YY1	Yin Yang 1

Chapter 1

1.1 Introduction

1.2 History

Von Willebrand factor is named after the Finnish doctor Erik von Willebrand (1870-1949). He was the first to document an unfamiliar bleeding disorder prevalent in families from Foglo, located on the islands of Aland in the Gulf of Bothnia. Although he couldn't determine the exact cause of the condition, he successfully differentiated it from hemophilia and other bleeding disorders [1, 2]. He provided an effective and comprehensive description of the medical and genetic characteristics of the disorder. Unlike hemophilia, the epitome of inherited bleeding disorders, both sexes were affected by this newly identified bleeding disorder, and the predominant symptom was mucosal bleeding. Prolonged bleeding time (BT) with a normal platelet count was the most important laboratory abnormality. A functional disorder of the platelets associated with a systemic injury of the vessel wall was suggested as a possible cause of the disorder. Initially, Dr. von Willebrand referred to the condition as hereditary pseudo hemophilia. Adding complexity to the situation, some authors labelled the disorder as vascular hemophilia. In the 1950s, it was revealed that this disorder was a result of a deficiency in a plasma factor rather than being attributed to platelet disorders [3].

During the 1970s, it was elucidated that the condition was attributable to a deficiency in a novel factor distinct from factor VIII. It was subsequently identified as the von Willebrand factor and the related disorder named von Willebrand disease (VWD). The VWF protein was purified

[4] and during the 1980s, the foundation for uncovering the molecular origins of the disorder was established with the cloning of the VWF gene [5-8]

1.3 Von Willebrand Factor

When a blood vessel is damaged at the site of injury, platelets, along with other clotting factors, aggregate to form a plug at the wound area. This plug seals the damaged vessel wall, preventing blood leakage. Numerous factors and multiple steps mediate blood clot formation. However, von Willebrand factor (VWF) serves as the initial and essential ingredient involved at the onset of this process [9, 10].

VWF is the plasma protein that assists and facilitates the activation of platelets, causing them to stick together and form a clump [11]. The expression pattern of this multimeric protein, with pro-coagulant properties, is highly limited to endothelial cells and megakaryocytes [12]. After VWF synthesis, it can be released into the bloodstream, deposited in the extracellular matrix of endothelial cells, or transported to storage organelles such as Weibel-Palade bodies (WPBs) in endothelial cells or α-granules in platelets [12]. In addition to VWF's primary function in hemostasis and thrombosis, VWF also functions as a carrier for a major coagulation factor, namely factor VIII, and increasing its half-life in circulation [13, 14].

In addition to these primary functions, VWF has been involved in several other biological processes. Notably, it has been documented to play a role in the metastasis of tumor cells, the process of angiogenesis, and various inflammatory responses [15-18].

1.4 Biosynthesis of von Willebrand factor

VWF gene is located on the short arm of chromosome 12 and includes approximately 180 Kb with 52 exons [6]. An 8 Kb mRNA is produced through transcription, forming a primary translation product consisting of 2813 amino acids, referred to as pre-pro-VWF [19, 20]. It contains a signal peptide of 22 residues (pre-peptide), a large pro-peptide of 741 amino acids, and a mature VWF protein with 2,050 residues [21, 22]. The basic VWF monomer is a 2050 amino acid protein with a molecular weight (MW) of 250 - 270 kDa [23]. Each monomer contains a number of specific domains with specific functions (Figure 1.4.1). The functions of each domain are summarized in Figure 1.4.1.

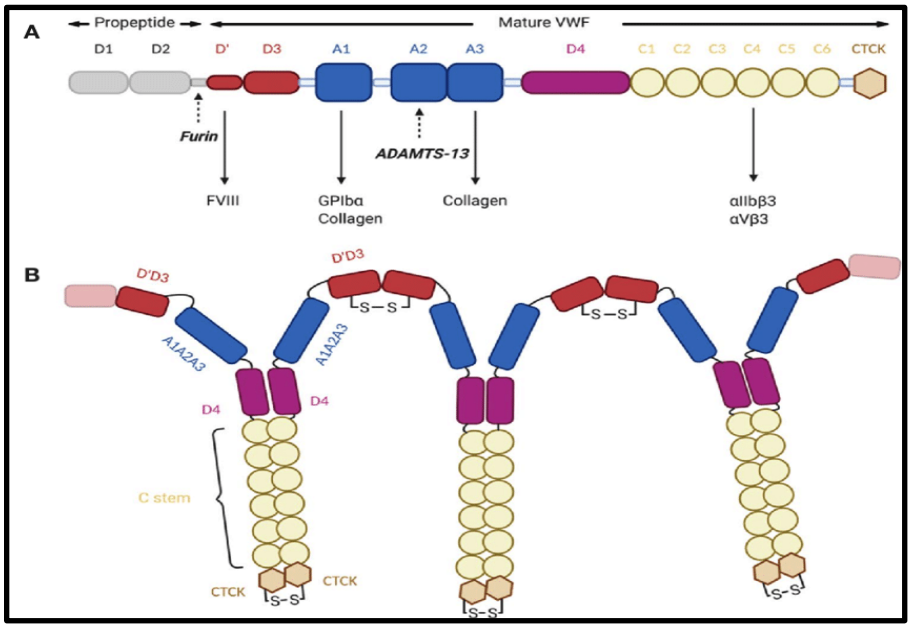

Figure 1.4.1. Structure of the von Willebrand protein with binding sites

A) Characterization of a mature VWF monomer. VWF is comprised of various domains (A, C and D) where specific protein binding sites or cleavage sites have been mapped. B) The architectural design of a VWF multimer. Multimers are linked via disulfide bonds that connect from head-to-head and tail-to-tail. The image reproduced from Risser et al [24].

The complex biosynthesis of VWF involves multiple posttranslational modifications. The signal peptide is removed within the endoplasmic reticulum (ER), and dimer formation of pro-VWF molecules occurs through disulfide bridges in the carboxyl-terminal region [25]. Then, VWF dimers are transferred to the Golgi apparatus. The pro-VWF dimers undergo posttranslational modifications, including glycosylation and sulfation, removal of the pro-peptide, and initiate multimerization to form very large multimers [26, 27]. Multimerization occurs via disulfide bonds that link the two N-terminal regions of each dimer [28, 29].

Following multimerization, VWF pro-peptide (also known as VWFpp) is cleaved from mature VWF by a paired amino acid-cleaving enzyme PACE (Paired basic Amino acid Cleaving Enzyme), also named furin [30, 31]. While mature VWF and VWF pro-peptide remain associated through noncovalent bonding with D′ D3 domain, they are stored in storage organelles, which include Weibel- Palade bodies and α-granules in endothelial cells and platelets respectively [32] (Figure 1.4.2).

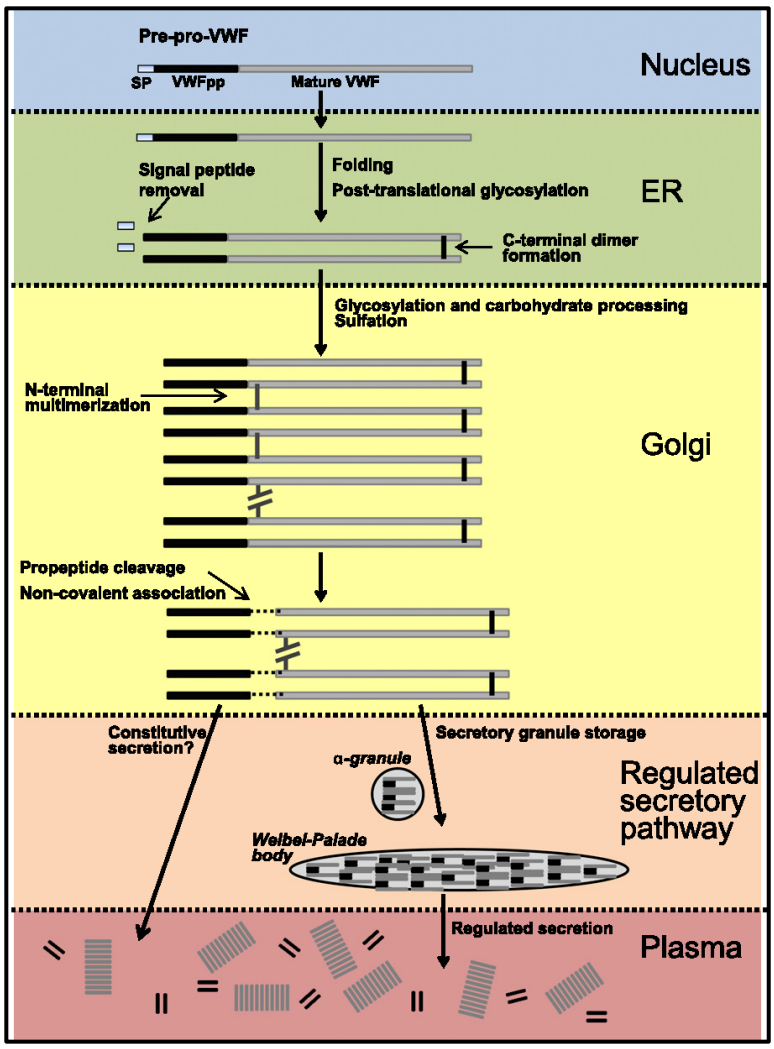

Figure 1.4.2. The process of VWF maturation

Pre-pro-VWF is transcribed as a primary product in the nucleus and after translocation to the endoplasmic reticulum (ER), it undergoes several posttranslational modifications including signal peptide removal, glycosylation, and dimer formation. Sulfation and multimerization occur in the Golgi and propeptide is cleaved from mature VWF but remains noncovalently connected. Finally, a high molecular weight multimer (HMWM) form of VWF is released into plasma through the regulated secretory pathway from storage organelles and propeptide and mature VWF separate and circulate independently from each other. Image reproduced from Haberichter et al [32].

1.5 Basal and regulated secretion of VWF

VWF in the plasma mainly comes from endothelial cells since platelets release their α-granule content only when activated [33]. However, two pathways are involved in the secretion of the VWF following synthesis in endothelial cells. One is a constitutive pathway in which immature VWF, mostly low molecular weight multimer, is secreted exclusively to the basolateral side of endothelial cells. The other one is from Weibel Palade bodies either through continuous release (basal release) or through the regulated pathway in the presence of a stimulus, which is the release of stored multimerized VWF from WPB [34].

The functional activity of VWF is directly proportional to its multimer size. It is known that multimers with high molecular weight are the most biologically active and exhibit the highest adhesion properties of VWF [35]. There are opposing views on whether most VWF is released from endothelial cells and comes from constant (constitutive) or controlled (regulated) release mechanisms [22, 36, 37]. However, a widely accepted opinion is that a significant proportion of VWF is constitutively released from the WPBs through the basal pathway [38].

Ultra-large VWF (UL-VWF) can be secreted into the plasma through the regulated pathway in response to various stimuli, such as hypernatremia, hyperglycemia, hypoxia, irradiation, and inflammation [39-43]. WPB exocytosis can also be triggered by other agents, such as thrombin, histamine, epinephrine, vasopressin, VEGF (vascular endothelial growth factor) and high levels of shear [44-48].

1.6 VWF and ADAMTS13

Like other plasma proteins, VWF's circulatory lifespan is limited. Its half-life in human plasma is approximately 16 hours [22]. Nevertheless, these values may fluctuate depending on individual health conditions and other contributing factors. However, due to differing

glycosylation levels on VWF molecules among individuals, the range may vary from 4.2 to 26 hours [22]. Different glycosylation patterns that affect VWF clearance can contribute to the observed alteration between individuals [49]. In particular, the existence of blood group ABO structures is known to be the major player in this observed variation [50]. There appears to be a protective effect associated with AB antigens against VWF clearance [51]. The plasma VWF levels of individuals with blood group O are 25% lower than those with other blood groups [52, 53].

As previously stated, the UL-VWF exhibits high activity and enhanced procoagulant function [54]. This is primarily due to its increased adhesive properties, facilitating platelet binding. UL-VWF is crucial for primary hemostasis. However, the unregulated expression of UL-VWF can lead to thrombotic complications.

In the presence of shear stress when UL-VWF is unravelled and the cleaving site (A2 domain) is exposed, ADAMTS13 (A Disintegrin And Metalloproteinase with ThromboSpondin type-1 repeats) can cleave UL-VWF multimers, at the Tyr1605-Met1606 peptide bond and consequently reduce the VWF prothrombogenic propensity [55] . ADAMTS13 is primarily secreted by stellate cells in the liver and, to a lesser extent, by vascular endothelial cells, megakaryocytes and platelets [55]. This ADAMTS13-mediated regulation of UL-VWF levels is necessary to prevent the formation of UL-VWF multimers and pathological platelet-VWF aggregates formation, which can block microvasculature. This is a common condition in patients suffering from thrombotic thrombocytopenic purpura (TTP) due to the lack of ADAMTS13 [55].

1.7 Von Willebrand Disease (VWD)

A deficit in the von Willebrand factor's quantity and/or quality contributes to the genetic bleeding disorder called von Willebrand disease (VWD)[56, 57] . Conversely, excessive circulating VWF is associated with cardiovascular mortality [58]. Mutation in the VWF gene in VWD patients alters VWF plasma concentration, structure and function [59]. There are three types of von Willebrand disease (type 1, type 2, and type 3) [57, 60].

Type 1 and type 3 VWD result from partial or complete quantitative deficiencies of VWF, respectively [61]. Patients with type 2 VWD experience a malfunction in the activity of VWF [61]. Type 2 is further classified into subtypes, 2A, 2B, and 2M, based on how impaired VWF interacts with platelets [61]. Additionally, a reduction in the ability of VWF to bind to FVIII causes subtype 2N [61]. In type 2N, resembling the scenario observed in mild hemophilia A, there is a reduction in FVIII level, resulting from impaired binding of VWF as a carrier to FVIII [61]. In type 2A, the von Willebrand factor exhibits a failure to attach to platelets, accompanied by a decrease in the presence of high molecular weight VWF multimers [62]. In type 2B, platelets firmly attach to high molecular weight von Willebrand factor (VWF), leading to elevated platelet clearance and reduced high molecular weight VWF levels [63, 64]. In type 2M, there is a reduction in platelet binding to VWF, and the level of VWF is decreased, while at the same time, the distribution of VWF multimer is normal [62, 65]. Type 2M is believed to occur due to the VWF protein's misfolding, resulting in diminished platelet adhesion and causing issues with collagen-binding ability [66, 67].

1.8 The role of VWF in platelet adhesion and aggregation

VWF plays a critical role in hemostasis and thrombus formation, contributing to platelet adhesion at injury sites and platelet-platelet cohesion or aggregation [68]. Upon endothelial cells activation, platelets are attached to the lesion site where subendothelial components might be

exposed [68, 69]. The interaction of platelets with the thrombogenic surface is known as adhesion, while aggregation is the subsequent growth of the hemostatic plug that relies on interplatelet interactions. Both features of platelet function are influenced by VWF interactions with specific platelet membrane receptors [68].

Multiple domains of VWF participate in the onset and expansion of platelet plugs. VWF is not an enzyme and, therefore does not possess enzymatic properties. VWF carries out its primary function in hemostasis by binding to other proteins such as factor VIII, receptors on the platelet surface, as well as components of the extracellular matrix [33].

The widely accepted theory of platelet adhesion mediated by the von Willebrand factor involves VWF binding to subendothelial collagen via its A1 and A3 domains. This binding facilitates the assembly of platelets along VWF's surface through reversible connections between VWF and glycoprotein Ib (GPIb) [70, 71]. The interaction between VWF and the glycoprotein Ib alpha (GPIbα) receptor on platelets is crucial for the initial attachment of platelets. This interaction is particularly significant under conditions of high blood flow and shear stress [70].

Platelet adhesion occurs when GPIbα binds to the A1 domain on tethered, unfolded VWF [72]. This binding triggers a signal that enables interactions between VWF and GPIIb/IIIa, leading to platelet stimulation and aggregation on the damaged vessel surface [73].

Platelet aggregation, which is the phase that results in forming a hemostatic plug within minutes, is facilitated by bond formation on the membranes of activated platelets. A key outcome of platelet activation is a change in the ligand-binding function of the platelet surface receptor αIIbβ3, also known as GPIIb–IIIa [74].

1.9 Association of genetic variations with VWF levels and functions

Plasma levels of VWF antigen vary widely among healthy individuals. This variability is influenced by environmental and lifestyle factors. However, around 60% of the variation can be attributed to genetic factors [75-77]. Single nucleotide variants (SNVs) in the coding, promoter regions, and introns of the VWF gene can change the levels of VWF antigen and activity in normal individuals [78, 79]. Genome-wide association studies (GWAS) have found sources of VWF variation heritability. The first comprehensive GWAS for VWF found VWF levels are associated with SNVs across eight loci, including VWF, ABO, STAB2, SCARA5, TC2N, STXBP5, STX2, and CLEC4M [80].

These variants could exert their effects through various potential mechanisms, such as changing mRNA splicing patterns, adjusting gene expression levels, or altering protein function [81]. It has been reported that genetic variations in ABO, STXBP5, and VWF have a strong correlation with the risk of venous thrombosis [82].

Studies are still ongoing to determine how these variants (that are identified by GWAS) affect VWF levels or function, but most VWF variants can be grouped into three types: genes that change VWF glycosylation (ABO) [53], secretion (STXBP5, STX2) [83, 84] from endothelial cells or platelets, and clearance from blood (STAB2, SCARA5, CLEC4M) [81, 84-86].

GWAS meta-analysis from the cohort of over 46,000 normal individuals of European, African, East Asian, and Hispanic descent identified eleven additional genome-wide associations significantly correlated with plasma VWF levels, including ARSA, C2CD4B, DAB2IP, FCHO2, GIMAP7, HLA-DGA1, OR13C5, PDHB, RAB5C, ST3GAL4, TAB1/SYNGR1 [87]. It seems that the primary function of most of these new loci is to regulate the secretion of VWF, but these associations require further confirmation and validation [81, 87].

1.10 VWF gene transcription

Transcription of the 178 Kb VWF gene is controlled by a complex mechanism, which has been studied through analysis and identification of the VWF promoter [88]. Our group has previously characterized those nucleotides −487 to +247 of the human VWF gene function as an endothelial-specific promoter in cultured cells [89]. The control of gene expression was found to be a complicated mechanism and needs complex interplay between multiple trans-acting factors with different DNA cis-acting elements in the promoter, which collectively regulates gene expression.

Several trans-acting factors have been identified as regulators of the VWF promoter. These factors can either activate or inhibit the transcription of the VWF gene (Figure 1.10.1). GATA6, NFY (interacting with the CCAAT sequence at position -18) and a histone H1-like protein (HLP) function as activators [89-92], while NFI and NFY (binding to a novel sequence CCGNNNCCC at position +226 to +234 next to the GATA binding site) function as repressors of the VWF promoter [90, 93]. Schwachtgen et al and Hough et al have identified Ets as another activator and Oct1, as well as E4BP4 as repressors of the VWF promoter [94-96]. Our lab has also demonstrated that the inhibition of VWF transcription involves a GATA6-NFY-HDAC association that occurs specifically in non-endothelial cells, resulting in recruitment of HDAC to the VWF promoter and thus leading to hypoacetylation of the histone H4 and transcription inhibition [91]. In conclusion, the result indicates that the expression of the endothelial-specific VWF genes is not determined by a single dominant factor but rather by a complex series of interactions.

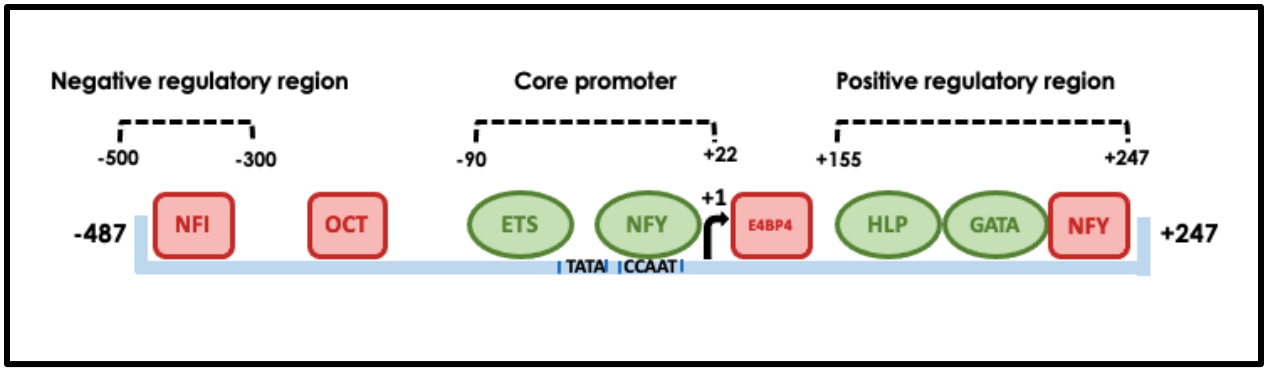

Figure 1.10.1. Transcription factor binding sites within VWF promoter sequences (-487 to +247)

Transcription factors acting as repressors (indicated in red) include Nuclear Factor I (NFI), octamer-binding transcription factor 1(OCT), nuclear transcription factor Y (NFY) and E4BP4. Transcription factors acting as activators (indicated in green) include E-Twenty-six (ETS), nuclear transcription factor Y (NFY), histone-like protein (HLP), and GATA-binding factor (GATA).

1.11 Organ-specific regulation of VWF promoter activity

The in vitro analyses that identified the endothelial-specific VWF promoter (sequences -487 to +247) were later followed by investigations in transgenic mice to determine the promoter's in vivo activation pattern. Investigation into the human VWF promoter in transgenic mice indicated that there are other regions of the VWF gene, that are crucial for activating the promoter in the endothelial cells of distinct organs (Figure 1.11.1). Our group in collaboration with Aird et al demonstrated that the 734 bp VWF promoter, extending from -487 to +247 sequences, function as a brain-specific VWF promoter sequence [97, 98] . It activates the expression of fused transgenes exclusively in the vascular endothelial cells of the brain in transgenic mice. Aird et al. subsequently identified the VWF promoter sequences that target activation specifically in the endothelial cells of the heart and muscle as well as brain [99]. Their group determined that to activate the expression of LacZ in the endothelial cells of the heart, muscle and brain of transgenic mice, additional

sequences of the VWF gene (2182 bp of the VWF 5′ flanking sequences, the first exon and the first intron) are necessary. We have also identified lung and brain-specific VWF promoter sequences [100]. Our study revealed that when sequences within intron 51 (referred to as I51HSS) of the VWF gene are placed either before or after the 734 bp VWF promoter (HSS-VWF-LacZ and VWF-LacZ-HSS), they activate the promoter in a subset of endothelial cells in the lungs as well as brain of transgenic mice [100]. The I51HSS cis-acting sequence exhibited a cell type specific functional interaction with the YY1 transacting factor containing complex [100]. These findings collectively demonstrate that specific segments of the VWF gene carry codes for gene expression in various vascular beds.

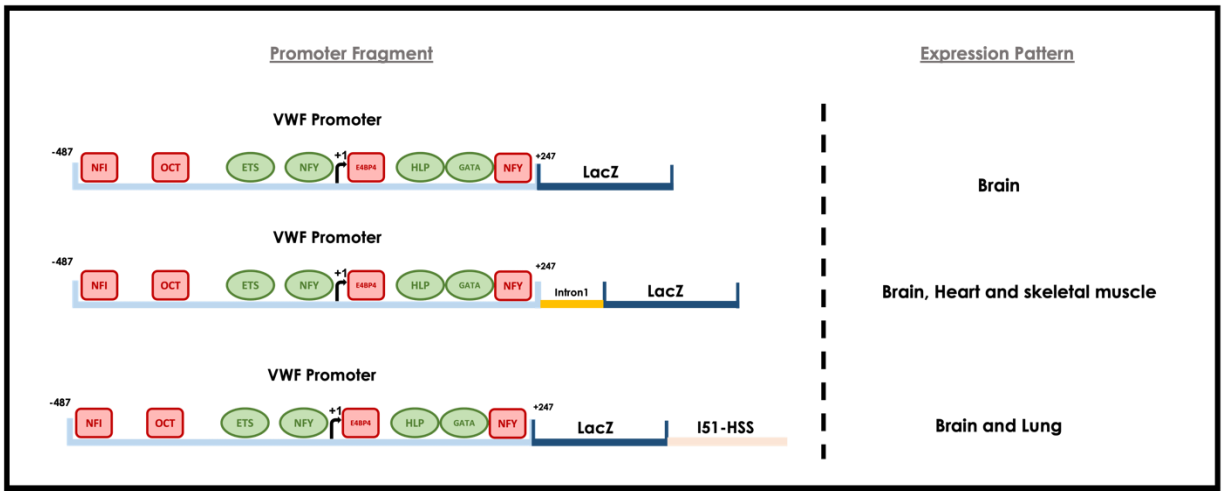

Figure 1.11.1. Organ specific VWF promoter sequences

1.12 The regulatory role of trans-acting factors in organ restricted VWF promoter activity

Our group has shown that repressors play a crucial role in maintaining the organ-specific regulation of the VWF promoter. We investigated the roles of NFI and NFY in this regulation in vivo. While the wildtype promoter was only active in the endothelial cells of the brain, mutation

at the binding site of NFI led to the activation of the VWF promoter in the endothelial cells of the heart and lungs as well as the brain [101] . Inhibition of NFY binding at the +226 region resulted in the expression of the mutant VWF promoter in brain and kidney endothelium [101]. The dual mutation at the binding sites of both NFI and NFY repressors resulted in the expression of the mutant VWF promoter in endothelial cells of all major organs, including brain, lung, heart, liver and kidney [101] (Figure 1.12.1). These results, combined with identification of distinct organ-specific regulatory regions, suggest that to turn on the activation of the VWF promoter, activators, located in distinct regions, must overcome the inhibitory effects of repressors such as NFY and NFI.

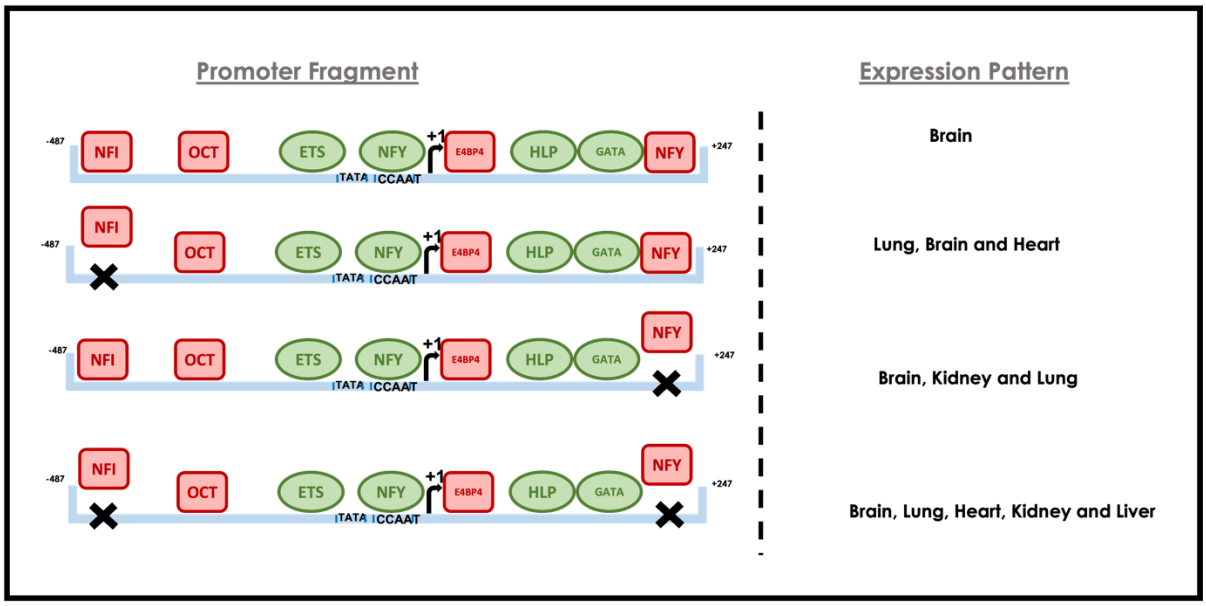

Figure 1.12.1. Activation of organ-specific VWF regulatory sequences in vivo

1.13 Regulation of VWF gene in endothelial cells and its response to inducers

Most inducers, including TNFα, IL-6, and DDVAP (desmopressin), activate the release of VWF from WPB into the bloodstream [102-104]. However, a few stimuli mediate the transcriptional upregulation of the VWF gene, such as irradiation and hypoxia [41, 42].

1.13.1 Mechanism of VWF gene regulation in response to irradiation

Previously, our analyses demonstrated that irradiation increases transcriptional activation of the VWF gene [42], and it is facilitated through the interaction of NFY with the CCAAT cis-element in the VWF promoter [105]. Moreover, in this process, the contribution of epigenetic modifications of VWF chromatin was observed. Our group had demonstrated that this mechanism involves a reduction in the association between NFY and HDAC1 and an enhancement in the association of NFY with PCAF [105, 106], which increases the recruitment of PCAF to the VWF promoter. This leads to elevated acetylation of promoter-associated histone H4 and consequently amplified transcription [106].

1.13.2 Mechanism of VWF gene regulation in response to hypoxia.

Endothelial dysfunction and phenotypic alterations significantly contribute to the pathology of various lung diseases, including pulmonary hypertension (PH) [107-109]. Hypoxia is a well-established contributor to the development of lung disease, including PH, and has been shown to induce the release of VWF [110]. By generating VWF-LacZ-HSS transgenic mice, which target the LacZ transgene expression specifically to the endothelial cells of the lung and brain, our group was able to investigate if conditions like hypoxia regulate the expression of the VWF gene, through the activation of the lung-brain specific promoter/regulatory sequences. Exposure of

transgenic mice to hypoxia demonstrated a significant increase in endogenous VWF mRNA and LacZ transgene expression in the lungs of hypoxic mice compared to control mice [41]. Moreover, they demonstrated that only in lungs (not in other organs) hypoxia-induced VWF was accompanied by alteration in the distribution pattern of the endogenous VWF and LacZ transgene expression from primarily large vessels (in control mice) to microvessels (in addition to large vessels) in lungs of hypoxic mice [41]. There were also significant increases in endogenous VWF mRNAs in brains, livers and hearts but not in the kidneys of hypoxic mice compared to the control [41]. They also explored the molecular mechanism of VWF response to hypoxia in cultured lung microvascular endothelial cells (LMECs). Hypoxia exposure of LMECs upregulated VWF mRNA and protein and this process was concomitant with decreased NF-IB binding and increased YY1 binding to their related binding sites on the VWF gene [41].

Additionally, hypoxia exposure increased the formation of platelet chains on the LMEC surface, as observed in the laminar flow adhesion assay [41]. This process was hindered by siRNA knockdown of either VWF or YY1 [41]. Subsequent studies expanded these analyses, showing that while heart endothelial cells also elevate VWF in response to hypoxia, the mechanism facilitating this process in the heart differs from that seen in lung endothelial cells. These findings suggest that endothelial cells from distinct organs respond differentially to hypoxia, based on the molecular mechanism that is employed in regulating the VWF gene [111].

1.14 Lung Transplantation

Lung transplantation is a surgical technique to replace diseased lungs by healthy lungs. Although lung transplants carry certain associated risks, lung transplantation has developed as an active option in treating a range of lung diseases, leading to extended life expectancy and

considerably improving quality of life. One of the serious complications of a lung transplant is thrombosis, which can occur at various times after transplantation [112-114]. The formation of a thrombus through transplantation procedure may cause allograft failure [114]. A prothrombotic state due to increased production of procoagulant molecules, such as VWF, is a significant risk factor for thrombus formation [115]. Previously in our research regarding the effect of hypoxia on VWF regulation, we demonstrated that VWF upregulation occurred in response to hypoxia. Therefore we hypothesized organs which are going to be transplanted undergo exposure to hypoxia for a period, this may lead to upregulated levels of VWF that could contribute to post transplant complications such as increased thrombogenicity. Additionally, we tested if modification in organ preservation method prior transplantation would moderate the hypoxia-induced VWF in lung transplantation.

1.15 Objectives

All the analyses regarding the effect of hypoxia on the regulation of VWF as an external stimulus prompted me to investigate: (i) the molecular mechanism of VWF gene regulation in response to aging as another inducer in various organs (Chapter 2-4) , (ii) the effect of potential hypoxia in organ transplant, specifically lung transplant procedure with regard to VWF expression and its contribution to transplant-associated thrombotic complications (Chapter 5).

2 Chapter 2

2.1 Age-Associated Increase in Thrombogenicity and Its Correlation with von Willebrand Factor

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A major contributing factor to aging-associated morbidity and mortality is the increased prevalence of thrombogenicity and consequently thrombotic disorders [116]. Thrombosis is the formation of blood clots within a blood vessel, and complications of thrombosis may cause acute life threatening or chronic conditions [117]. Thrombotic events in large vessels, such as coronary arteries and cerebral blood vessels, could lead to acute catastrophic events, for instance, heart attack and stroke respectively. Moreover, microvascular thrombosis may contribute to the target organ(s)' suboptimal functioning as a result of perfusion hindrance. Microvascular thrombosis is the hallmark of a number of vascular diseases, including thrombotic microangiopathy, disseminated intravascular coagulation, and sickle cell disease among others [117, 118]. Although many genetic and environmental risk factors contribute to thrombotic events in the general population, aging is a universal and non-modifiable risk factor for thrombosis [119]. Epidemiological studies have indicated an association between age and higher risk incidents of thrombotic cardiovascular complications, including acute myocardial infarction, stroke, and venous thromboembolism [120-122]. Aging considerably increases the incidence of venous and arterial thrombotic events, with an annual rate of venous thromboembolism (VTE) from 1 per 10,000 in young, to 1 in 100 among the elderly population over the age of 80 [123]. It has been reported that after the age of 45, the incidence of atherothrombotic brain infarction and myocardial infarction doubles after every 10 years of life [122, 124]. The cause of this sharp increase in thrombosis with aging is not yet clearly understood. The dramatic increase in thrombogenicity in the elderly population may reflect the pathophysiology of aging. Aging-associated development of molecular and anatomical abnormalities have been detected that could contribute to this process. These include increase in diameter and thickness of vessel walls, fragmentation of internal elastic lamina, and vascular smooth muscle cells hypertrophy within the vessel walls that results in

endothelial injury, which is a key factor in promoting thrombosis [125-128]. Aging is also associated with the development of a proinflammatory phenotype, a well established risk factor for thrombosis [129-131]. Furthermore, several clinical studies have reported that aging alters the levels of various circulating and vessel wall associated factors, leading to an imbalance in the ratio of procoagulants to anticoagulants in favour of procoagulants, and consequently a prothrombotic state [132, 133]. Procoagulant protein von Willebrand factor (VWF) is among factors that display an age-associated increase in circulation [134]. VWF is fundamental to the process of thrombus generation since it initiates the first step of the process, specifically adherence of platelets to endothelial/subendothelial surfaces [135]. It also mediates the subsequent step of platelet-platelet interactions that is necessary for the formation of platelet aggregates [135]. The circulating levels of VWF are not only elevated with aging in healthy population, but are also influenced by multiple pathologies associated with the process of aging, such as insulin resistance, obesity, and various risk factors that are associated with cardiovascular disorders [136, 137]. Thus, it stands to reason that increased production of VWF, without a proportional increase in anticoagulant proteins, may contribute significantly to increased thrombogenicity with aging [131, 134]. In this review, we aim to discuss the impact of aging on the VWF levels, its consequences under physiological/pathophysiological conditions, and potential mechanism(s) that may instigate aging associated elevation of VWF.

2.1.1 VWF Role in Physiology

VWF is an adhesive multimeric glycoprotein that plays a pivotal role in normal hemostasis and thrombosis [138]. It was first identified by Dr. Eric von Willebrand during the treatment of a severe bleeding disorder [1]. The gene coding for VWF is located on chromosome 12, spans 180 kb and contains 52 exons [6, 139]. VWF expression is strictly restricted to two cell types, endothelial cells (ECs) and megakaryocytes (precursor of platelets) [139]. VWF biosynthesis is a complex process that starts with the translation of an approximately 9 kb VWF mRNA to generate a precursor protein that is subjected to a wide range of post-translational modifications. These include dimerization, multimerization, and cleavage, in addition to extensive glycosylation and sulfation [22, 140]. Cleavage of the precursor protein generates two components, propeptide (VWFpp) and mature subunit (VWF), which remain noncovalently associated until secreted into the bloodstream [22]. Multimerized VWF is stored in specialized organelles known as Weibel-Palade bodies (WPB) and alpha granules in endothelial cells and platelets, respectively [22]. While alpha-granules release VWF upon activation of platelets, endothelial cells release VWF by two pathways, namely constitutive and regulatory secretions. Dimer/smaller multimers of VWF are secreted continuously from endothelial cells (consecutive secretion), while most biologically active ultra large WPB stored multimers (UL-VWF) are secreted through regulatory pathway in response to inducers [141]. Biosynthesis, structure, processing, and secretion of VWF have been subjects of many excellent reviews periodically [22, 141, 142]. VWF that is secreted from endothelial cells either enters circulation or is deposited in the subluminal extracellular matrix, which when exposed in response to injury, provides the binding sites for platelets [117, 121]. Due to its highly adhesive nature that allows association with various extracellular matrix proteins, circulating VWF can also interact with other proteins (such as collagen) in exposed extracellular

matrix at the site of vascular injury [143]. When the shear rate is elevated, globular VWF is unfurled and its various binding sites are exposed, mediating a transient interaction between VWF and platelet [23]. This leads to initial platelet captures and slows platelets movement, which is then stabilized through the interaction of various other proteins, in addition to VWF (Figure 2.1.1) [144, 145]. Once initially captured platelets are stabilized and activated to release their VWF, VWF and other proteins on adherent activated platelets provide recruitment and attachment sites for additional circulating platelets [76, 81]. Activated platelets provide a major site for prothrombin complexes assembly and enhance thrombin generation, thus directly participating in thrombosis. This physiological function of subluminal VWF is critical for maintaining vessel integrity while the repair of the vessel wall proceeds at the site of injury. Upon completion of the repair process, VWF cleaving enzymes, mainly metalloproteinase ADAMTS13 (although other proteases that target VWF, i.e., ADAMTS28, have also been identified [146]), as well as other thrombolytic enzymes, mediate the disassembly of platelet aggregates, leading to thrombus resolution and restoration of unobstructed blood flow (Figure 2.1.1). However, VWF is also shown to bind to the endothelial cell membrane in the intact luminal surface [147]. Membrane bound VWF could also mediate platelet adhesion to the intact endothelial surface under permissive conditions (Figure 2.1.1), and consequently, this may be a contributing factor towards undesired thrombus formation [147].

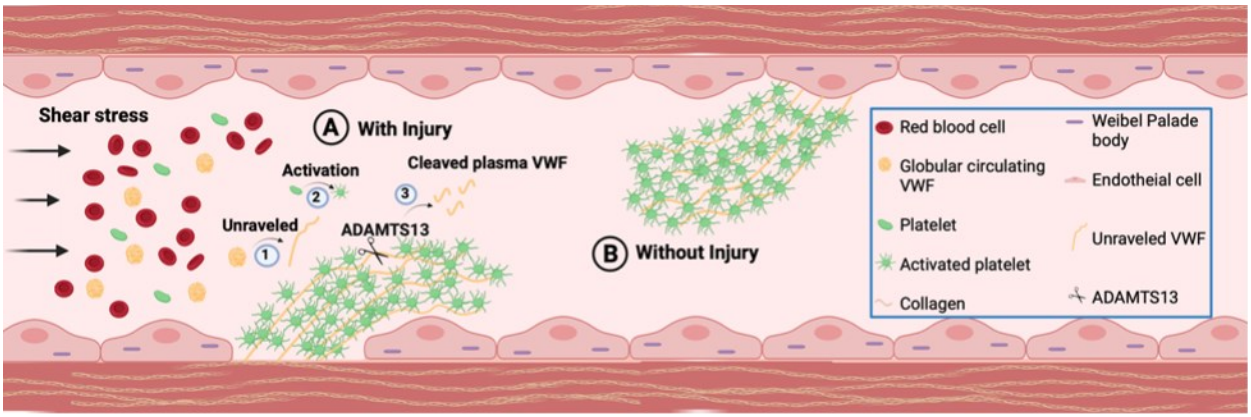

Figure 2.1.1. VWF Function

VWF is stored in Weibel Palade bodies (WPB) and deposited in extracellular matrix of vascular endothelial cells, as well as circulating in plasma as globular form. (A) At the site of injury in presence of shear stress globular VWF attach to the exposed subendothelial collagen layer and (1) unravel to form the most adhesive form of VWF. The extracellular matrix deposited VWF is similarly exposed to bind platelets. Platelets captured by unraveled VWF are (2) activated and form VWF-platelet aggregates to seal the damaged vessel wall and prevent blood leakage while repair. Following the completion of repair process ADAMTS13 (Scissors) will (3) cleave UL VWF to smaller VWF multimers to resolve platelet aggregates. (B) External stimuli induce secretion of VWF, which may bind to endothelial luminal surface membrane and mediate platelet aggregate formation through a similar process as that observed at the site of vascular injury.

VWF also performs additional hemostatic functions by binding and stabilizing FVIII (a vital coagulation protein of the intrinsic clotting pathway) in circulation. Furthermore, VWF has been recently shown to participate in a number of other physiological and pathophysiological processes, such as inflammation, angiogenesis, and cancer metastasis, which are the subject of many recent excellent reviews and will not be discussed here [15, 148-150]. Plasma levels of VWF are influenced by a combination of environmental, genetic, and pathological factors, although what is considered as normal levels constitute a wide range (50–200%) in a healthy population [81]. It is estimated that about 30% of the variation in the plasma VWF levels is related to genetic factors

through pedigree analyses, while twin studies have suggested this value to be 65% [76, 77, 81, 151]. Another major contributing factor to the variation in circulating VWF levels is the ABO blood group, which is proposed to account for approximately 30% of the observed variability [53, 81]. Blood Group O individuals have 25% lower plasma VWF levels than non-O blood group individuals [50, 53]. The presence of AB antigens coincides with a protective function against VWF clearance. This is proposed as the main reason for higher VWF levels in the non-O blood group; although recent evidence suggesting a significantly decreased levels of cellular VWF protein in lung endothelial cells of the O blood group individuals compared to non-O have also been presented [152].

2.1.2 Coagulopathies Associated with von Willebrand Factor

Considering the central role of VWF in hemostasis and thrombosis, both decreased and increased levels of VWF (outside the normal range) are associated with coagulopathies. Deficiencies of VWF, either quantitative or ve, are associated with the most common inherited bleeding disorder known as Von Willebrand disease (VWD) [153-155]. There are four main types of VWD based on phenotypic analysis of the VWF: Type 1, Type 2, Type 3, and platelet-type [62, 156, 157]. The most common and mildest form of VWD is Type 1 (quantitative) that is caused by low VWF production or accelerated clearance [157]. Type 2 VWD (qualitative) is characterized by defects in VWF (abnormal function), and it accounts for 20–40% of VWD cases [62]. Whereas Type 3 VWD is a total quantitative deficiency of VWF and is the most severe form of VWD that represents 5% of VWD cases [157]. In platelet-type VWD, a gain of function mutation within platelet surface receptor (glycoprotein Ib alpha) for VWF leads to exaggerated platelet-VWF interaction, resulting in thrombocytopenia and, consequently, prolonged bleeding time [156]. In

contrast to VWF deficiency, increased plasma VWF levels and functional activity is a significant risk factor for thrombosis [158]. The thrombotic event may involve large vessels, leading to acute consequences such as stroke or heart attack [159]; or implicate microvasculature, leading to various chronic diseases such as thrombotic microangiopathy [160]. Significantly elevated levels of VWF are observed in thrombotic thrombocytopenic purpura (TTP), which results from a profound deficiency of ADAMTS13. It is the leading cause of thrombotic microangiopathy characterized by the formation of platelets aggregates in arterioles, capillaries, and venules [161, 162]. Several other pathological conditions including vascular dementia [162, 163], Alzheimer's disease [163, 164], traumatic brain injury [165], acute respiratory distress syndrome [166], and kidney failure [167] are also associated with increased plasma levels of VWF. Such disorders may arise or be exacerbated potentially as a consequence of increased microvascular occlusion.

2.1.3 Influence of Aging on VWF and ADAMTS13 in a Healthy Population

Age-associated increases in plasma levels of VWF have been reported in studies using animal models [168-170], but also well established in the human population (Table 2.1-1). A study of 74 centenarians and 110 individuals aged 45–86 years old has shown that VWF antigen levels, and its functional activity, were markedly elevated in centenarians compared to the younger cohort [171]. The study did not find any significant difference between Blood Group O and non-O [171]. Another cross-sectional study on a cohort of 207 individuals aged 1–87 years demonstrated that the levels of circulating VWF increased significantly in the elderly (with an overall increase of 1.56-fold), and that the increase was more significant in the non-O blood group individuals [172]. Although the influence of the ABO blood group was not significant in young individuals, it became more pronounced with advancing age [172]. Furthermore, the age-related increase in VWF levels

was accompanied by a 2.03 fold increase in VWF functional activity in older individuals [172]. This increase in VWF levels was followed by simultaneous elevation in FVIII coagulant activity and antigen levels (reaching up to 1.5 fold by later life in healthy individuals) [172, 173]. A recent cross sectional study of 2923 individuals also reported elevation of VWF:Ag as well as FVIII coagulation activity per decade of age after adjustment for comorbidities including body mass index, inflammation and hormone use; and the increase was higher for non-O blood group individuals [173]. However, the influence of aging and plasma VWF levels on FVIII level and coagulant activity in mild Hemophilia A patients is scarcely studied. One study reported a significant positive correlation between FVIII coagulant activity and aging without amelioration of bleeding [174]; whereas another study identified only minor influence of aging on FVIII levels/activity [175]. A cross sectional study of 3616 Japanese participants, aged 30 to 79 years reported that VWF levels tend to increase (significantly influenced by ABO blood groups), whereas ADAMTS13 activity was decreased (not significantly influenced by ABO blood groups) with advancing age, leading to an increased ratio of plasma VWF levels to ADAMTS13 activity with aging in both men and women [176]. It is worth noting that this study reported a lower level of ADAMTS13 activity in men compared to women, suggesting that elderly males are at a higher risk of developing hypercoagulable state and consequently thrombosis [176]. Another study of the healthy Arab population revealed that VWF levels increase with aging, while ADAMTS13 antigen levels remain unaffected but ADAMTS13 activity decreases with aging [177]. Inconsistent findings have been reported concerning the impact of biological sex on rising VWF levels with aging. The large study of blood donors in South Wales (n = 5052) reported that there was a minor but significant difference between VWF levels among men and women (approximately 114 IU dL^(-1) for men vs. 109 IU dL^(-1) for women) [177]. In contrast, the study of the healthy Arab

population found lower plasma levels of VWF in females compared to males, but only in Blood Group O females [177]. ADAMTS13 activity and antigen levels were unaffected by blood groups in both genders of the Arab population [177]. Collectively these reports suggest that the trend of rising VWF levels with aging is similar in both women and men. While it is reported that women have a higher rate of increase in the VWF plasma levels during middle age (41–50 years), this may be attributed to changes in hormone levels during pregnancy, menstrual cycle, hormone based contraceptives, and menopause, all of which can influence VWF levels [178-180]. From several studies, it is clearly demonstrated that circulating VWF levels tend to increase gradually with aging in humans, at a 1–2% rate per year in adults, but the absolute increase is greater with advancing age [176, 181, 182]. However, a higher rate of elevation in VWF levels with age occurs after midlife (above 40 years of age) in both men and women [136, 178]. It is reported that among different variables such as age, gender, blood type, and use of medications, only age was significantly predictive of VWF level, and exhibited age-related elevation [178]. Since VWFpp and mature VWF have different half lives (2 h and around 8–12 h for each respectively), VWFpp levels have been used as a marker for endothelial cell activation and VWF release [102, 172]. VWFpp and mature VWF are cleared independently, hence multiple studies have utilized the ratio between VWFpp and VWF antigen (VWF:Ag) levels to evaluate the synthesis and clearance of VWF [183, 184]. These analyses have demonstrated that in addition to upregulated VWF secretion, a reduction in the ratio of the VWFpp:VWF antigen is observed with aging, suggesting an age-associated decrease in VWF clearance and consequently its increased half life in circulation [172]. However, a report of immunohistochemical analysis of cellular VWF expression in lung tissues of 64 pulmonary neoplasia patients [26 children (mean age 3.5 years) and 38 adults (mean age 55 years)], demonstrated a significantly elevated levels of cellular VWF in arterioles,

capillaries, and venules of adult lungs compared to children [185]. This observation suggests that in addition to increased secretion and decreased clearance, a potential increase in VWF biosynthesis, specifically in smaller vessels, maybe a significant contributing factor to the elevated VWF levels in response to aging. Furthermore, another potential source of the age-associated increase in VWF may involve alteration in platelets activity and consequently increased release of VWF from platelets alpha granules. For instance, aging is associated with increased content of basal phosphoinositide in the platelet plasma membrane [186], which is important for activating the second messenger signaling pathway and triggering platelet activity. This suggests that aging may alter platelet transmembrane signaling pathways and consequently its propensity for activation and as a result platelet aggregate formation. Furthermore, studies have shown an age-related reduction in nitric oxide (NO) levels [187-189], contributing to abnormal platelet aggregation. Collectively, procoagulant alterations in endothelial cells' phenotype and platelets activities with aging, may contribute to enhanced VWF levels and decreased ADAMTS13 activities, providing the foundation for thrombosis.

Table 2.1-1. Effect of age on Von Willebrand factor in the healthy population.

Location	Population characteristics	Results	Author
Italy	184 healthy individuals included 74 centenarians (100–107 years), 55 younger controls (<45 years), 55 older controls (>45 years)	centenarians VWF:Ag level 245 (U/dL) in O blood group and 285 (U/dL) in non-O blood group; older controls VWF:Ag level 99 (U/dL) in O blood group and 152 (U/dL) in non-O blood group; younger controls VWF:Ag level 77 (U/dL) in O blood group and 115 (U/dL) in non-O blood group; VWF activity level centenarians > older controls > younger controls	Coppola et al., 2003[171]
Canada	207 healthy individuals included 113 old (55–87 years), 42 middle-age (30–49 years), 52 young (1–17 years)	Plasma VWF level increased with age reaching a 1.71-fold by old age in non-O and 1.25-fold in O blood group. VWF activity reached 2.03-fold by old age and VWFpp level (as a marker of VWF secretion) elevated to 1.26-fold in older individuals with blood type non-O than blood type O.	Albanez et al., 2016[172]
United Kingdom	Cohort of 5052 healthy individuals	VWF:Ag level increase minor and non-significant up to age of 40 years but elevated significantly above age>40 years. For the whole cohort absolute increase between each age group: 2 IU/dL between <20 years and 21–30 years, 3 IU/dL between 21–30 years and 31–40 years, 7 IU/dL between 31–40 years and 41–50 years, 8 IU/dL between 41–50 years and 51–60 years, and 15 IU/dL between 51–60 years and 61–70 years.	Davies et al., 2012 [178]

Japan	Cohort of 3616 healthy Japanese population between the age range of 30–79 years	VWF:Ag increased with advancing age and the linear regression coefficient being 1.37 and 1.30 in men and women respectively. Plasma ADMATS13 activity decreased with age, significantly after 60 years and regression coefficient was −0.642 resulted in increased ratio of VWF:Ag to ADAMTS13 activity with age.	Kokame et al., 2011 [176]
Kuwait	200 healthy individuals	Plasma VWF level significantly higher in older individuals and ADAMTS13 activity decreased with age, however, ADAMTS13 level not affected by age.	Al-Awadhi et al., 2014 [177]
Netherlands	Cohort of 2923 individuals between the age range of 18–70 years	VWF:Ag increase per decade of age 18 IU/dL and for FVIII activity 12 IU/dL. After adjustment for acquired factors (comorbidities, body mass index, reduced kidney function, hormone use, and inflammation), the increase per decade 13 IU/dL for VWF:Ag and 9 IU/dL for FVIII activity. Increase was higher in blood group non-O.	Biguzzi et al., 2021 [173]

2.1.4 Impact of Aging on von Willebrand Disease

The normal reference range of plasma VWF levels is between 50 and 200 IU/dL in a healthy population [190]. However, as described in the previous section, quantitative (Types 1 and 3) and qualitative (Type 2) deficiencies of VWF occurs that result in von Willebrand disease (VWD), characterized by excessive mucocutaneous bleeding. Although VWF levels in the range of 30–50 IU/dL have been generally considered as an indication of VWD, currently this range is considered as a low normal VWF level, which is nevertheless a risk factor for developing bleeding disorders [190]. The observation of an age-associated increase in plasma VWF levels of a healthy population suggests that aging may also impact elderly VWD patients. While Type 2 and Type 3 VWD are

diagnosed due to qualitative deficiencies or severe reductions in VWF levels, diagnosing and differentiating mild forms of VWD—Type 1 from low levels of VWF is more challenging. Regardless of classification into normal but low levels, or Type I mild VWD, this population might be a target group that could benefit from the age-related rise in VWF levels. Consistent with this hypothesis, VWF levels were shown to rise with aging in milder forms of VWD, but not in severe forms, such as Type 2 and 3 VWD [137, 191, 192]. Furthermore, normalization of VWF levels with aging may affect symptoms, severity, and diagnosis, in mild forms of VWD. A retrospective study of 126 patients with Type 1 VWD or 'low VWF' indicated a significant increase in VWF levels and activity in elderly VWD Type 1 patients, leading to complete VWF levels normalization in 27.8% of participants, although amelioration of bleeding symptoms was not shown [193]. Another retrospective study of Type 1 VWD patients aged 16–60 for eleven years found normalization of VWF antigen levels, activity, and FVIII levels in 18 patients out of 31 [194]. Additionally, Willebrand in the Netherlands (WiN-) study of 664 VWD patients aged 16–85 years found that with aging VWF levels fell within the normal reference range in mild Type 1 VWD, while remained unchanged in Type 2 VWD [195]. Increased VWF levels were also followed by elevated VWF activity and FVIII levels in Type 1 VWD, but not in Type 2 VWD [195]. Very few studies have been performed involving Type 2 VWD patients, whereas none of the studies have included Type 3 VWD patients. This may reflect the expectation that qualitative deficiencies associated with Type 2 VWD will not be ameliorated with increased levels; and that the nature of extensive deficiency associated with Type III VWD (i.e., gene deletion), is incompatible with potential increases in VWF levels. To evaluate the effect of comorbidities in VWD patients, a cross sectional study was performed that included 536 VWD Type 1 and Type 2 patients aged 16–83 year [137]. It was found that hypertension, diabetes mellitus, thyroid dysfunction and cancer were

associated with increased VWF levels in Type 1 VWD compared to VWD patients without these comorbidities, whereas no association was found in Type 2 VWD patients [137]. Lowered VWFpp:VWF antigen ratio indicated that reduced clearance of VWF in circulation was a potential cause of elevated VWF levels in these comorbidities [137]. It is vital to investigate and establish the effect of aging on VWD patients since it has serious consequences on the treatment of VWD generally, as well as in conjunction with other age-related comorbidities. A recent study has described in detail the impact of aging on VWF levels and presented an in depth discussion of its association with bleeding symptoms in VWD patients [192]. However, despite accumulating evidence in support of age-associated increase in VWF levels and function, whether this results in amelioration of bleeding symptoms in VWD patients remains unclear. The uncertainty and conflicting reports are proposed to be due to differences in the study designs and procedures, patients' characteristics, or presence of comorbidities. It is also hypothesized that with aging there may be a requirement for higher levels of VWF for optimal hemostasis; thus, amelioration of bleeding symptoms may not be observed in aging VWD patients [192].

2.1.5 Potential Mechanisms of Age-Associated Increase in VWF

An increase in plasma levels of VWF may result from increased regulated and/or constitutive secretion. Numerous factors including thrombin, histamine, nitric oxide (NO), calcium, vasopressin, epinephrine, leukotrienes, superoxide anions, sphingosine-1-phosphate, endothelin-1 (ET-1), prostacyclin and shear stress were shown to affect VWF plasma levels [58, 196-200]. While some factors may alter the regulation of VWF secretions, others may affect VWF levels by altering VWF gene transcription/post transcriptional activity [197-199]. A combination of alterations in secretion and production levels may contribute to increase circulating VWF levels.

Although the underlying mechanism of age-associated increase in VWF remains unclear, age-related alterations in the circulatory and vascular physiology/ pathophysiology could provide some insights. Elevated VWF levels with aging may be an intrinsic property of aged endothelial cells, may occur in response to various age-related vascular dysfunctions, and/or increased inflammation. Aging generally leads to the development of a proinflammatory phenotype in the elderly population [201, 202], and inflammation is a major contributing factor to increased thrombogenicity [203]. Pathogenicity of inflammation induced thrombosis is complex, and interactions between inflammation and thrombosis is a bidirectional process: inflammation promotes platelets aggregation and coagulation, while activated platelets and coagulation factors promote inflammation [201, 202]. Vascular injury is the key factor that associates inflammation with thrombosis; however, inflammation induced thrombosis may also develop without endothelial injury [202]. Elevated levels of pro inflammatory markers have been reported in many older individuals, even in the absence of pre-existing health conditions or related risk factors [204, 205]. Observation of elevated inflammatory mediators, leading to chronic low grade inflammation in the aged population, is known as “inflammaging”; and it promotes endothelial dysfunction [204]. VWF plasma level is known as a useful marker for endothelial dysfunction and may be relevant to the disease process. An age-related alteration in endothelium and surrounding microenvironment may also contribute to age-associated increase in VWF levels and thrombogenicity. Aging alters the levels of vasodilator NO and vasoconstrictor endothelin1 (ET-1), which are both regulators of VWF secretion [206]. Nitric oxide (NO) inhibits exocytosis of WPB and platelets’ alpha granules; hence, it inhibits VWF release [58, 207]. Aging is shown to result in decreased NO bioavailability and sensitivity to NO, thus ameliorating/reducing its’ inhibitory effect on exocytosis and consequently promoting increased VWF secretion from storage

organelles [208, 209]. In contrast, ET-1 that increases VWF expression in endothelial cells, and shown to cause elevated circulating VWF levels when infused in men [210], exhibits an elevated levels with aging. Aging also affects circadian rhythm and is associated with fragmentation or loss of rhythm [208]. VWF promoter activity is under direct regulation of major circadian transcriptional regulators CLOCK/Bmal1 [211]. Bmal1 deficiency not only directly increases VWF mRNA and protein levels, but also downregulates nitric oxide synthase (eNOS) activity and consequently decreases NO production; thus, indirectly contributing to VWF release as well [211, 212]. However, it should be noted that the reported age-associated decrease in Bmal1 expression was based on analyses of the master circadian pacemaker in the suprachiasmatic nucleus (SCN), the hippocampus, and cingulate cortex [213]. Further studies are required to determine whether aging similarly downregulates Bmal1 in other tissues/cell types, including endothelial cells to directly establish that decreased Bmal1 with aging increases VWF levels. Another potential contributor to the age-related increase in VWF could be related to alteration in osmolarity. In vivo studies in mice demonstrated that water restriction and elevated plasma sodium concentration, above normal physiological range, significantly increased VWF mRNA and protein levels [39]. This process was shown to be mediated through binding of the tonicity regulated transcription factor NFAT5 to the VWF promoter [39]. With aging water intake decreases and the risk of dehydration increases. Numerous studies on the elderly have reported that aging decreases perceived thirst and water intake in response to hypovolemia, hypertonicity, and hormonal stimulus [214-216]. Thus, dehydration in older people increases sodium concentration and this could lead to an increase in VWF production as well as secretion. In addition to increased VWF levels, aging is also reported to be associated with a decrease in the level and activity of VWF cleaving enzyme ADAMTS13 [171, 176]. Thus, a combination of these two events cumulatively

contributes to the generation and persistence of ultra large VWF multimers in circulation [176, 217]. Under the condition of high shear stress, ADAMTS13 mediates increased proteolysis of high molecular weight (HMW) [218], but aging is reported to be also associated with reduced shear stress, thus further contributing to a condition that promotes persistence of high molecular weight VWF in the circulation and elevated platelets aggregation [219]. Together these age-associated alterations directly and/or indirectly increase VWF production, secretion, and stability, potentially leading to the generation of platelets aggregates and consequently increased thrombotic events (Figure 2.1.2).

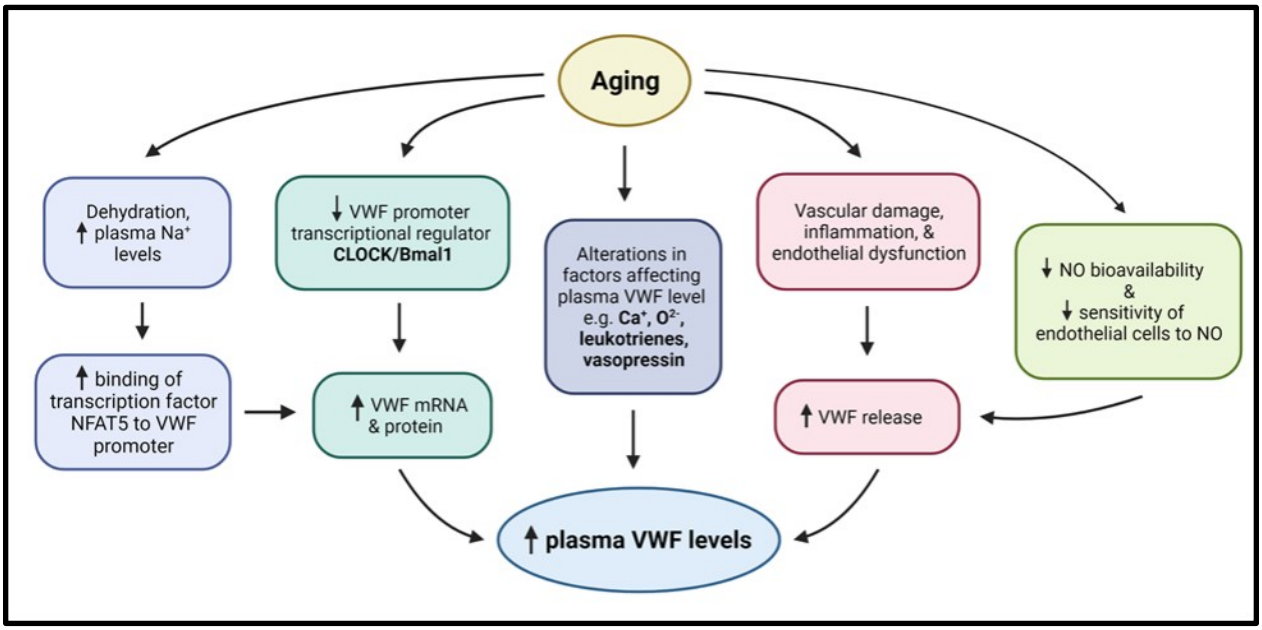

Figure 2.1.2. Potential mechanisms of age associated increase in VWF levels

Upward and downward arrows depict increase and decrease respectively.

2.1.6 VWF and COVID-19

Various infectious diseases including HIV [220], malaria [221], scrub typhus [222], and Dengue virus [223] infection have been associated with thrombosis and increased circulating VWF

levels. The COVID-19 disease caused by the SARS-CoV-2 (Cov-2) virus starts with an early infectious stage, but it may be followed by viral pneumonia and systemic inflammation that could potentially lead to respiratory failure and multiple organ dysfunction [224-227]. It is now well established that the COVID-19 is also associated with hypercoagulation and severe thrombotic complications "Corona virus-associated coagulopathy" (CAC) [228-230]. Several studies have reported incidence of ischemic stroke, deep vein thrombosis, pulmonary thromboembolism, and thrombosis in small brain vessels leading to cerebral microbleeds in 20–30% of COVID-19 patients admitted to the intensive care unit (ICU). The incidences of these coagulopathies are significantly higher among aging population of COVID19 patients and encompass more than 50% of elderly non-survivors [231-234]. Cov-2 infection is reported to facilitate vascular inflammation (vasculitis) and enhance the prothrombotic state, potentially leading to organ dysfunction due to the presence of microangiopathy and microthrombi in vascular beds of multiple organs [235-237]. Multiple hematological abnormalities are observed in COVID-19 patients including elevated levels of fibrinogen and D-Dimer in critically ill patients [229, 238, 239]. However, hematological analyses of these patients have also found that VWF levels and activities were elevated five to six folds beyond the upper normal limit [230, 240]. In a cohort of 150 elderly Covid-19 patients with a medical history of cardiovascular, respiratory, and multiple organ abnormalities, median VWF levels were reported at 455% of normal [230]. In some cases, VWF elevation is augmented by a deficiency of the VWF cleavage enzyme ADAMTS13 or reduced ADAMTS13 activity in the blood [228, 241, 242]. Considering the age-associated increase in VWF, it is plausible to hypothesize that elevated basal levels of VWF in elderly may increase their susceptibility towards development of CAC complications as a result of Cov-2 infection. Consistent with this hypothesis are the observations that microangiopathy in the lung is highly prevalent among COVID19 patients

with CAC [227, 243-245], and that age-associated increase, specifically in cellular expression of VWF, is observed in lung microvasculature [185]. Cov-2 infection is not only associated with increased plasma levels of VWF but also was reported to have a qualitative impact on the VWF activity, specifically enhancing its adhesive properties, such as collagen binding [242]. While more investigation is required, emerging data suggest that COVID-19 is associated with increased consumption of HMWM VWF [242, 246, 247], which could be indicative of increased platelets aggregate formation. Consistent with this, lower platelet counts were observed in some COVID19 patients, which could be due to hyperactivation of platelets and aggregate formation following platelet interaction with VWF [230, 248]. Regarding ADAMTS13 levels in COVID-19 patients, the reports are less consistent. Some studies reported normal levels of ADAMTS13 while few reported decreased levels of ADAMTS13 [228, 249, 250]. There have also been reports of a significantly reduced ratio of ADAMTS13/VWF, which suggest that while ADAMTS13 may or may not be reduced, a very high concentration of VWF could overwhelm the capacity of ADAMTS13 [247]. It is also noteworthy that neutrophil extracellular traps (NETs) are found in the microvascular thrombi present in the heart, lung, or kidney of COVID-19 patients [251]. It is reported that contents released from NETs might reduce enzymatic activity of ADAMTS13, consequently increasing VWF levels [252]. Summary of the literature reports of investigation into the levels and activities of VWF and ADAMTS13 in critically ill COVID-19 patients with and without coexisting comorbidities is presented in Table 2.1-2.While the mechanism for increased VWF levels in COVID-19 patients with the hypercoagulable state remains unclear, it is noteworthy that elevated VWF levels are predominantly reported in critically ill patients with health conditions/comorbidities that are also associated with increased VWF levels, including elderly and/or those with hypertension and diabetes mellitus. Elevated VWF levels associated with various

chronic diseases and natural aging may generate a heightened prothrombotic state. This, combined with CoV-2 induced increase in VWF levels, could significantly tip the balance of pro- and anti-thrombotic state in favor of thrombosis. CoV-2 may directly infect endothelial cells, and/or indirectly stimulate endothelial cells to release VWF as a result of enhanced inflammatory environment. The lungs were reported as the main target of Cov-2, which can bind to Angiotensin converting enzyme 2 (ACE2), on the alveolocytes [253]. ACE2 receptor, which belongs to the renin angiotensin system and has a major role in regulating blood pressure, is also expressed on the endothelial surfaces of the heart, lung, kidney, and systemic vessels [254]. ACE2 is proposed to mediate Cov-2 entry into endothelial cells [255, 256], independently, or potentially in combination with other proteins, which have been also identified as receptors for Cov-2 on endothelial cell surface [257]. However, interaction of virus particles with ACE2 may also lead to a loss of ACE2 enzymatic function and consequently decrease plasma level of vasoprotective angiotensin [258-260]. This in turn could result in acute phase inflammatory responses leading to endothelialitis (vascular endothelial inflammation) and hypersecretion of VWF [17, 261, 262] . Proinflammatory conditions arising as a result of cytokine storm generation further contribute to prolonged endothelial stimulation and activation of complement cascade; both of which stimulate VWF release [17, 261, 262]. Inflammation, endothelial injury, and dysregulated immune response instigate assembly of membrane attack complex (MAC) on endothelial cells, which increases endothelial cytosolic Ca2+ concentration that stimulates VWF release from Weible Palade bodies [263]. Inflammatory cytokine TNF-alpha can regulate VWF expression indirectly by decreasing NO synthesis through inhibition of eNOS expression in endothelial cells [264, 265]. Consistent with this, significantly decreased NO levels has been reported as a result of Cov-2 infection [266, 267]. Additionally, significantly increased IL-8 and IL-6 inflammatory cytokines also promote

VWF secretion in a concentration dependent manner. Furthermore, IL-6 has been reported to also decrease ADAMTS13 activity, which further increases accumulation of ultra large VWF levels with higher activity [103].

Table 2.1-2. VWF and ATDAMTS13 levels and activity reported in critically ill COVID-19 patients with and without pre-existing comorbidities.

Normal reference VWF antigen range (%) 42–136[a]. Normal reference VWF activity range (%) 42–168[b]. Normal ADAMTS13 antigen reference range (%) 40–130[c]. Normal ADAMTS13 activity reference range (%) 50–150[d]. The value is reported as median (interquartile range)

Location	Patient Characteristics (n)	Mean Age (years)	VWF Antigen (%)[a] Mean (min–max)	VWF Activity (%)[b] Mean (min–max)	ADAMTS13 (%)[c] Mean (min–max)	ADAMTS13 Activity (%)[d] Mean (min–max)	Author
Italy	Intubated COVID-19 patients (11)	–	529 (210–863)	387 (195–550)	–	–	Panigada et al., 2020 [248]
Netherlands	COVID-19 patients admitted to ICU (12)	61.8	408.0	374.0	48.0	–	Huisman et al., 2020 [268]
France	Patients with radiological signs of interstitial pneumonia (212)	63.9	361.0	–	–	–	Rauch et al., 2020 [269]
Germany[e]	Covid-19 patients with high severity (150)	63.0	455 (350–521)	328 (212–342)	–	–	Helms et al., 2020 [230]
Italy[e]	Covid-19 patients with high severity (19)	59.0	476 (381–537)	388 (328–438)	–	55 (42–68)	Mancini et al., 2021 [246]
United Kingdom[e]	Intubated ICU patients (24)	65.0	350 (302–433)	–	–	–	Ladikou et al., 2020 [270]
Ireland	Covid-19 patients admitted to ICU (28)	55.0	690.2 (467–848.4)	–	–	–	Ward et al., 2021 [271]
France[e]	Patients with critical form of Covid-19 in ICU (89)	62.0	507 (428–596)	399 (333–537)	–	–	Philippe et al., 2021 [272]
France[e]	Covid-19 patients with Venous thromboembolic events (38)	63.0	522 (411–672)	–	–	59 (38.8–70.5)	Delrue et al., 2021 [273]
Italy	Intubated ICU patients (6)	62.0	634 (455–772)	450 (339–496)	37.3 (24–56)	–	Morici et al., 2020 [250]
United States	Intubated ICU patients (48)	64.0	565.0	390.0	–	–	Goshua et al., 2020 [240]
Italy	Patients with Covid-19 pneumonia (37)	61.8	280.8	265.1	–	–	Taus et al., 2020 [274]

Spain	ICU patients with the history of hypertension and diabetes mellitus (22)	68.0	368.6	–	–	38.9	Marco et al., 2021 [275]
Italy[e]	Patients with novel coronavirus pneumonia (10)	61.0	324.1	341.5	69.0	–	De Cristofaro 2021 [276]
Spain[e]	Covid-19 patients with cardiovascular disease and diabetes (23)	64.0	306.0	–	47.3	–	Blasi et al., 2020 [277]
Germany	Covid-19 patients with mild to moderate severity (75)	66.0	403.0	–	67.8	–	Doevelaar et al., 2021 [247]
Italy	Patients with the history of hypertension and diabetes (19)	69.0	331.4	321.7	–	–	Ruberto et al., 2020 [278]
United States[e]	Covid-19 non-survivors (90)	72.5	441.0	321.0	–	48.8	Sweeney et al., 2020 [279]
Spain[e]	Severe Covid-19 patients (50)	–	355 (267–400)	–	53.2 (38.8–65.3)	–	Rodríguez et al., 2021 [280]
Italy	Covid-19 non-survivors (9)	72.0	395.5	–	32.2	–	Bazzan et al., 2020 [228]
Belgium	Covid-19 patients admitted to ICU (9)	57–64	475.0	429.0	45.0	–	Hardy et al., 2020 [281]
United States[e]	17	42–58	448 (362–529)	313 (190–347)	–	–	Masi et al., 2020 [282]
Sweden[e]	Patients with Covid-19 high care (12)	53–69	425 (321–465)	–	57 (42–62)	–	Meijenfeldt et al., 2021 [283]
Germany	Covid-19 non-survivors (5)	78.0	260.4	217.6	–	43.3	De Jongh et al., 2021 [284]
France[e]	Covid-19 patients admitted to ICU (22)	–	456 (402–493)	355 (297–416)	458 (364–615)	–	Pascreau et al., 2021 [285]

Another factor that may also contribute to elevated levels of VWF in COVID-19 patients is development of hypoxic conditions. COVID-19 patients may suffer from acute lung injury that causes acute respiratory distress syndrome (ARDS) and decreases tissue oxygenation, leading to hypoxic condition. Hypoxia was shown to be a mediator of VWF release from WPB [110]. Furthermore, we have previously demonstrated that hypoxia also induces VWF transcriptional upregulation, and consequently contributes to increased platelet aggregates formation [41, 111]. Specifically, in the lungs of mice, hypoxia altered the VWF expression pattern, leading to

expression of VWF in a significant proportion of lung microvascular endothelial cells that generally do not exhibit VWF expression under normoxic conditions [41]. Thus, COVID-19 induced hypoxia may upregulate VWF levels, and this has been supported by the clinical observation of rising VWF levels with increased oxygen requirements in a few COVID-19 patients [269]. Additionally, it is noteworthy that elevated VWF levels has been reported only in COVID-19 positive pneumonia patients [276]. This suggests that synergistic effects of hypoxia with other COVID19 related conditions might be responsible for increase in VWF levels during the disease progression. Thus, pre-existing factors, such as aging and comorbidities, in combination with hypoxic, immunologic, and proinflammatory alterations that ensue in COVID-19 infection, may collectively contribute to a significant increase in VWF levels and consequently increased thrombogenicity. COVID-19 does not affect all individuals in a similar manner, and this is also reflected in development of hypercoagulability and thrombosis. This, however, is consistent with highly variable basal levels of VWF in population [81], which may play a role in pre-disposing certain individuals to increased risk of CAC development. For instance, individuals with high basal levels of VWF, when aged and/or have comorbidities, if infected with Cov-2 may reach a VWF threshold that leads to the development of thrombosis, while people with low basal levels of VWF may not. Consistent with this hypothesis, some studies have indicated that COVID-19 patients with Blood Group O (25% lower VWF levels than other blood groups) are significantly underrepresented in thrombogenic complications [286, 287]. Similarly, Type I VWD patients with significantly lower VWF levels are also underrepresented in groups of COVID-19 patients with thrombogenic consequences. These observations suggest that determining the level and activity of VWF in COVID-19 patients may provide a useful prognostic marker towards COVID-19

morbidity and mortality as well as identifying patient populations that are at the risk of developing thrombotic complications.

2.1.7 Conclusions

Aging is associated with increased risk of arterial, venous, and microvascular thrombosis in the elderly population. Elevated levels of VWF in circulation, as well as cellular levels, are also reported with aging. Considering the central role of VWF in thrombus formation, a likely association between this rise in VWF and thrombogenicity is conceivable. Various external factors, specifically alterations in inflammatory and immunological landscape may significantly contribute to the increased production of VWF with aging. However, age-associated increase in VWF occurs in healthy population and exhibits a gradual constant increase. Thus, it is also conceivable that inherent alterations in endothelial cell phenotype may occur with aging that culminates in increased production of VWF. Such possibility may be specifically important in relation to microvascular endothelial cells, which do not normally exhibit VWF expression. Testing of this hypothesis will provide significant insights towards determining the underlying molecular basis of age-associated increase in VWF. It will also contribute to potential development of targeted therapies to combat various age-associated macro- and micro-vascular diseases in elderly population with and without comorbidities. Therapeutic approaches that are developed or under consideration include strategies to interfere with binding of platelets to VWF, or reduction in the levels of ULVWF multimers. For instance, some clinical treatment options include blocking of VWF and platelet interaction using anti VWF antibody such as AJW200 [288-290] or a humanized anti-VWF antibody fragment (nanobody) caplacizumab, which bind to VWF A1 domain [291, 292]. In addition, anfibatide, a snake venom derived compound that is an antagonist of GPIb receptor on platelets, can inhibit platelet-VWF interaction [293, 294]. Reduction in the

levels of ULVWF may be achieved through restoration/increase of ADAMTS13 function by introduction of recombinant ADAMTS13 (rADAMTS13) [295] or through the action of N-acetylcysteine (NAC) that disrupts disulfide bonds in the VWF A1 domain [296, 297]. In addition, aptamers that are DNA or RNA single-stranded nucleic acids, which bind to their target protein with high affinity and specificity, could present as novel potential therapeutic agents. DNA aptamer TAGX-0004 [298] and RNA aptamer BT200 [299] are inhibitors of the VWF A1 domain that may be considered as potential inhibitors of VWF function.

2.1.8 Author Contributions:

Conceptualization, N.J., P.A. and A.M.R.; literature survey, P.A. and A.M.R. writing—original draft preparation, P.A. and A.M.R.; writing—review and editing, N.J.; supervision, N.J. funding acquisition, N.J. All authors have read and agreed to the published version of the manuscript.

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2.1.11 Conflicts of Interest:

The authors declare no conflicts of interest.

3 Chapter 3

3.1 Aging is associated with organ-specific alterations in level and expression pattern of von Willebrand factor

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3.1.1 Abstract

BACKGROUND: VWF (von Willebrand factor) is an endothelial-specific procoagulant protein with a major role in thrombosis. Aging is associated with increased circulating levels of VWF, which presents a risk factor for thrombus formation.

METHODS: Circulating plasma, cellular protein, and mRNA levels of VWF were determined and compared in young and aged mice. Major organs were subjected to immunofluorescence analyses to determine the vascular pattern of VWF expression and the presence of platelet aggregates. An in vitro model of aging, using extended culture time of endothelial cells, was used to explore the mechanism of age-associated increased VWF levels.

RESULTS: Increased circulating plasma levels of VWF with elevated levels of larger multimers, indicative of VWF functional activity, were observed in aged mice. VWF mRNA and cellular protein levels were significantly increased in the brains, lungs, and livers but not in the kidneys and hearts of aged mice. Higher proportion of small vessels in brains, lungs, and livers of aged mice exhibited VWF expression compared with young, and this was concomitant with increased platelet aggregate formation. Prolonged culture of endothelial cells resulted in increased cell senescence that correlated with increased VWF expression; VWF expression was specifically detected in senescent cultured endothelial cells and abolished in response to p53 knockdown. A significantly higher proportion of VWF expressing endothelial cells in vivo exhibited senescence markers SA-β-Gal (senescence-associated β-galactosidase) and p53 in aged mouse brains compared with that of the young.

CONCLUSIONS: Aging elicits a heterogenic response in endothelial cells with regard to VWF expression, leading to organ specific increase in VWF levels and alterations in vascular tree pattern of expression. This is concomitant with increased platelet aggregate formation. The age-associated

increase in VWF expression may be modulated through the process of cell senescence, and p53 transcription factor contributes to its regulation.

3.1.2 Introduction

Among all age-related health complications, cardiovascular disease is the most prevalent cause of death in elderly population [300]. In addition to the age-associated alterations that lead to thickening and stiffness of vascular tissues, impairment of the endothelial cell function is a major contributor to the increased risk of thrombosis and its consequences, including heart attack and stroke [301]. Moreover, aging is also associated with microvascular dysfunction such as that observed in the brains of patients with vascular cognitive impairment (VCI) [302, 303] . Vascular thrombosis can occur in hypercoagulable state as a result of high ratio of the procoagulant molecules, such as von Willebrand factor (VWF), to those with anticoagulant functions including VWF cleaving enzyme ADAMTS13 [304]. VWF is a multimeric adhesive procoagulant protein, which under physiological conditions, is exclusively expressed in endothelial cells and megakaryocytes, although recently some cancer cells of non-endothelial/megakaryocyte origin were also shown to express VWF [305, 306] . The critical role of VWF in hemostasis and thrombosis is well established [307, 308], while recently additional roles for VWF in angiogenesis, inflammation and cancer metastasis have also been described [18, 149, 306, 309-312]. The critical role of VWF in maintaining haemostasis is based on its function as primary mediator of platelets adhesion [25]. At the site of injury, VWF binds to exposed sub endothelial layer and mediates platelets adhesion to seal the damaged site and prevent blood leakage during the repair process [313]. VWF functional activity is highly correlated to its multimers size. High molecular weight multimers (HMWM) presenting with increased platelets and collagen binding sites are most

effective in mediating platelets adhesion and wound healing process [314, 315]. Following the completion of the repair process and reestablishment of vascular integrity, VWF multimers are cleaved by enzymes, mainly ADAMTS13, to initiate disaggregation of the platelets plug and restore blood flow [316, 317]. However, in the absence of injury, endothelial cells can also release VWF in response to stimuli, such as irradiation and hypoxia, as well as inflammation or other conditions that lead to endothelial dysfunction [41, 42, 110, 318]. Under these circumstances, released VWF can bind to endothelial cell surfaces and promote undesired platelets adhesion and aggregation, which may lead to thrombus formation [319]. VWF also contributes to regulation of thrombotic process as a result of its other major function as a carrier and stabilizer for coagulation factor VIII [86]. Considering the central role of VWF in haemostasis and thrombosis, as well as its participation in inflammation, angiogenesis and metastasis, it is not unexpected that dysregulated VWF expression could contribute to pathological conditions [310, 311]. Indeed, the most common inherited bleeding disorder, von Willebrand disease (VWD) is caused by insufficient levels or defective structure of VWF [2, 154, 320], whereas excessive plasma levels of VWF are a risk factor for cardiovascular mortality [321, 322]. Several studies have demonstrated that aging is associated with increased plasma levels of VWF [136, 172, 191, 194, 195, 217, 323, 324]. Analysis of VWF levels in a cohort of 207 normal individuals demonstrated a 1.56-fold increase in VWF levels of aged (mean 71+/-7) compared to young (7+/-5) 33. This increase was independent of the blood type, which is a well-documented factor influencing VWF levels [172]. Although, increased circulating levels of VWF may reflect degranulation of Weibel-Palade bodies (VWF storage organelles in endothelial cells [47]), its' continuous and sustained increased levels may reflect alterations in the process of VWF gene transcription [325]. Here we used a murine model to explore whether age-associated increase in circulating plasma levels of

VWF is correlated with increased VWF transcripts, whether the process exhibits organ-specific characteristics, its potential functional consequences with regard to platelet aggregate formation, and finally whether cellular senescence is a potential underlying mechanism contributing to this process.

3.1.3 Material and methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

3.1.3.1 Animals, organ harvest and blood sample collection

The study was approved by the Health Sciences Animal Policy and Welfare Committee at the University of Alberta. Blood was collected from male young (3 months) and aged (18-19 months) C57BL/6NCrl mice into EDTA (anticoagulant)-treated tubes (Vacutainer, BD sciences). Cells were removed from the plasma by centrifugation of the whole blood for 15 minutes at 1,000x g using a refrigerated centrifuge. The supernatant was immediately transferred into tubes and stored at –80°C. VWF concentrations in mice plasma were determined using mouse VWF ELISA Kits (MyBioSource; Elabscience) respectively, according to the manufacturer's protocol. ADAMTS13 concentrations were determined using mouse ADAMTS13 ELISA kit (Abbexa). Organs (brains, hearts, lungs, livers and kidneys) from young and aged mice were harvested and either snap frozen in liquid nitrogen (for RNA and protein quantification), soaked in 30% sucrose overnight at 4^{o}C and embedded in Tissue-Tek optimal cutting temperature (OCT) medium, or fixed in 10% formalin and paraffin embedded. For all animal experiments "n" represents the number of mice per group.

3.1.3.2 VWF multimer analysis

Electrophoresis and western blotting of SDS-agarose gel electrophoresis were carried out as previously described with minor modifications [326]. Briefly, the electrophoresis gel constituted 1.6% (w/v) main, and a stacking gel 0.8% (w/v) of high gelling temperature agarose (Seakem® HGT(P) agarose, Lonza) in running gel buffer (0.1 M Tris, 0.1 M glycine, and 0.4% (w/v) SDS, pH 8.8). The electrophoresis was performed for approximately 2 h at 10 mA per gel. Then the gel was soaked in transfer buffer [containing 1 mM 2-mercaptoethanol (2-ME), 48 mM Tris, 39 mM glycine, 20% methanol, and 0.037% SDS, pH 9.2 (not adjusted)], and incubated for 30 min at room temperature. Thereafter, the gel was washed using transfer buffer without 2-ME for 15 min at room temperature. VWF multimers were transferred onto 0.2-µm pore nitrocellulose membranes by semi-dry transfer system set at 23 V for 30 min. Non-specific sites on the membrane were blocked by overnight incubation in 5% non-fat dry milk in washing buffer (PBS, pH 7.4, containing 0.1% Tween 20) at 4 °C. Following washing steps, the membrane was incubated with the primary antibody (rabbit anti-human VWF antibody, diluted 1:1000 in incubation buffer) for 2 h at room temperature. Membrane was washed three times and incubated with the secondary antibody [Goat anti-Rabbit IgG H&L (HRP), diluted 1:5000 in incubation buffer (PBS, pH 7.4, containing 0.1% Tween 20)] for 1 h at room temperature. Images were acquired by GE ImageQuant LAS 500 Imager.

3.1.3.3 Quantitative real-time PCR and Western blot analyses

VWF and p53 mRNA and protein levels were determined by quantitative real-time PCR (qRT-PCR) and Western blot analysis respectively, as previously described [101, 106]. Relative mRNA expression levels of VWF and p53 were calculated by the ΔΔCt method, using the

housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT) mRNA for normalization as previously described [41]. The oligonucleotide sequences of the primers used are included in the Supplementary Materials (Table 3.1-1). Primary antibodies against VWF (Abcam), p53 (Santa Cruz) and tubulin (Millipore Sigma), and secondary antibodies goat anti-rabbit IgG H&L (HRP) (Abcam) or goat anti-mouse IgG (Millipore Sigma) were used for Western blot analyses. Protein bands were detected using the GE ImageQuant LAS 500 Imager. The results were analyzed by ImageJ for signal intensity quantification.

3.1.3.4 Immunofluorescent and confocal microscopy analyses

Tissue cryosections (5μm thickness) were subjected to immunofluorescence staining using primary antibodies against VWF (sheep anti VWF antibody, Abcam), CD31 (rabbit anti CD31 specific antibody, Abcam), CD41 (rat anti CD41, Abcam), p53 (mouse anti p53, Santa Cruz), and β-galactosidase (rabbit anti β-galactosidase, Biorbyt) and appropriate secondary antibodies [For the list Please see the Major Resources Table in the Supplemental Materials (Table 3.1-3)] as previously described [41]. Images were taken using a WaveFX confocal microscope from Quorum Technologies using Volocity software and analyzed by ImageJ software. Samples stained only by secondary antibodies (without primary antibodies) were considered as negative controls (Figure 3.1.9)

3.1.3.5 Acetylsalicylic acid treatment

Acetylsalicylic acid (ASA) (Sigma Aldrich) was dissolved in sterile saline. Aged mice were weighed and administered either a low dose of ASA (10mg/kg) or an equivalent volume of sterile saline every 12 hours (total of 4 treatments) by oral gavage. Forty-eight hours after initiation

of treatment mice were euthanized and brains were harvested, frozen in OCT and subjected to immunofluorescence analyses as described above.

3.1.3.6 Imaging Flow Cytometry: SA-β-Gal Staining and VWF Staining

Human umbilical vein endothelial cells (HUVECs) were maintained for 0-3 weeks in culture to reflect the aging process and subjected to fluorescence activated cell sorting (FACS) analysis as follows. Cells were stained for SA-β-Gal and analyzed via imaging flow cytometry using the method described by Biran et al.[327, 328] with slight modifications. Cells were lifted, washed, and fixed with 2% paraformaldehyde for 5 minutes. Cells were then washed with 1mM $MgCl_2$/PBS (pH 6) twice, before resuspending in SA-β-Gal staining buffer: 1mg/ml X-Gal (Sigma Aldrich), 5mM $K_3[Fe(CN)_6]$ (Sigma Aldrich), 5mM $K_4[Fe(CN)_6]\cdot 3H_2O$ (Sigma Aldrich) in 1mM $MgCl_2$/PBS (pH 6). Cells were incubated for 8 hours in a 37˚C incubator with no CO_2 in the dark, and then washed twice with flow cytometry buffer (PBS with 2% FBS and 0.09% NaN_3) and then fixed/permeabilized using eBioscience Intracellular Fixation and Permeabilization Kit (Invitrogen). Cells were fixed for 30 minutes at room temperature and then washed twice with permeabilization buffer. Sheep anti-VWF antibody (Abcam) were diluted 1:100 in permeabilization buffer and incubated overnight at 4˚C with constant rotation. Cells are then washed twice with flow cytometry buffer and have donkey anti-sheep Alexa Fluor 647 secondary antibody (Invitrogen) added for one hour at room temperature. Cells were washed and nuclei were stained with DAPI (ThermoFisher Scientific), after which cells were resuspended in 50 μL of flow cytometry buffer and run on the Amnis ImageStream Mark II imaging flow cytometer. Cells were gated based on area vs aspect ratio to identify single cells, then captured based on a positive nuclear stain. SA-β-Gal staining intensity was determined via the mean pixel intensity of the bright field

channel (lower values = higher stain intensity); typically, a value of -100 to -150 represents the upper cut-off for senescent cells. At least 10,000 events were captured before data was analyzed on the IDEAS imaging software.

3.1.3.7 Knockdown using siRNA

HUVECs at 70-80% confluency were transfected with 20 nM of nonspecific siRNA (NSsiRNA) or p53siRNA (Ambion) using Lipofectamine 2000 (Thermo Scientific) according to the manufacturer's instructions. siRNA treatments were performed twice (48 hours apart) and 48 hours after the second treatment cells were collected for RNA and protein analyses. For week 3 samples, cells were treated at the beginning of week 3 and collected after the second treatment by the end of week 3.

3.1.3.8 Statistical analysis

Data were analyzed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA). Data normality distributions and homogeneity of variance were analyzed by Shapiro-Wilk tests and *F* test, respectively. Two-group comparisons wherein all data sets passed normality and homogeneity of variance tests were performed by Student *t*-test; otherwise, 2-group comparisons were performed by Mann-Whitney U test. The Welch correction was applied to the *t*-test analysis of the data that passed normality test but did not pass the equal variance test. For sample size less than 5 per group, it is recommended either to perform a non-parametric statistical test or to use a larger sample size. However, in the current study, parametric test has been performed with n=3 which is our study limitation. Multigroup comparison were performed either by one way or two way ANOVA followed by Tukey or sidak post hoc test. All used statistical analysis are provided

in the legends of each figure. Data are presented as the mean ± standard error of the mean (SEM) or median±interquartile range wherever was appropriate. For all statistical tests, a p-value less than 0.05 was considered significant.

3.1.4 Results

3.1.4.1 VWF protein and mRNA levels increase with age in mice in an organ specific manner

We aimed to investigate the mechanism of age-associated increase in VWF levels using mice as a model organism. Thus, we first proceeded to determine whether age-associated alteration in circulating levels of VWF that is reported in humans is also observed in mice. We explored plasma levels of VWF in male young and aged mice. Alterations in VWF levels are reported in response to sex hormones, i.e. estrogen [17, 329]. Thus, to exclude the effect of hormonal interplay on the analysis of the results, we only included male mice for all the studies described here. Plasma levels of VWF were higher in aged mice compared to their younger counterparts (Figure 3.1.1 A). These data demonstrated that age-associated increase in VWF is not limited to humans and occurs similarly in mice. Since, circulating levels of the VWF may be influenced by the levels of VWF cleaving enzyme ADAMTS13, which contribute to its clearance [316], we also checked the plasma levels of ADAMTS13 in young and aged mice. The result demonstrated that there was no significant difference in the plasma levels of ADAMTS13 between young and aged mice (Figure 3.1.1 B). These results demonstrate that consistent with that observed in human, in mice also there was an age-associated increase in VWF levels, while there was no significant alteration in ADAMTS13 circulating levels.

Since functional activity of VWF is directly dependent on the size of its multimers, and the high molecular weight multimers (HMWM) are known to be the most hemostatically active [330], to determine the potential for functional activity of the circulating VWF in the aged and young mice, we performed the VWF multimerization analysis. Plasma from young and aged mice were subjected to VWF multimers analysis as described in "Methods" and the result clearly demonstrated an increase in HMWM in the plasma of aged compared to young mice, indicative of increased circulating levels of functional VWF (Figure 3.1.1 C).

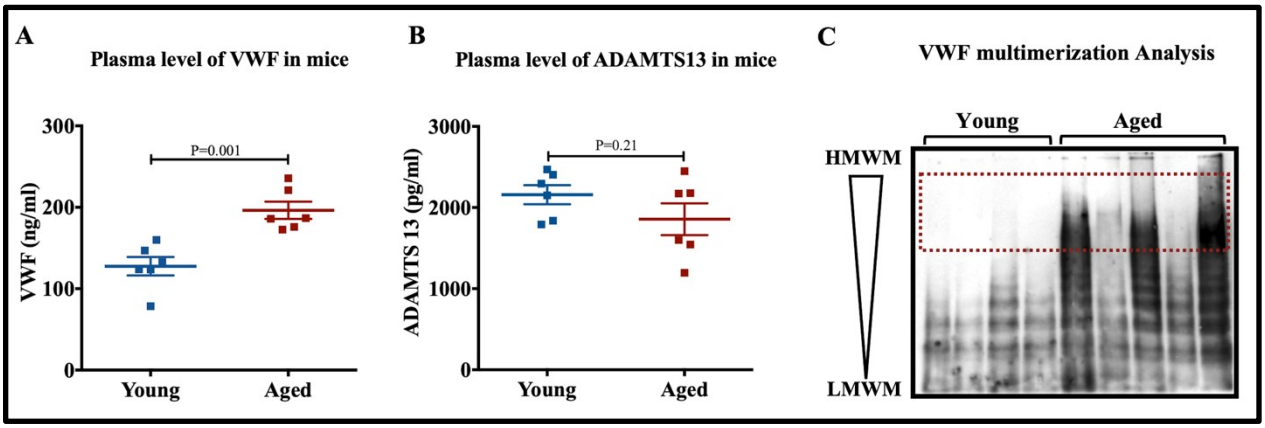
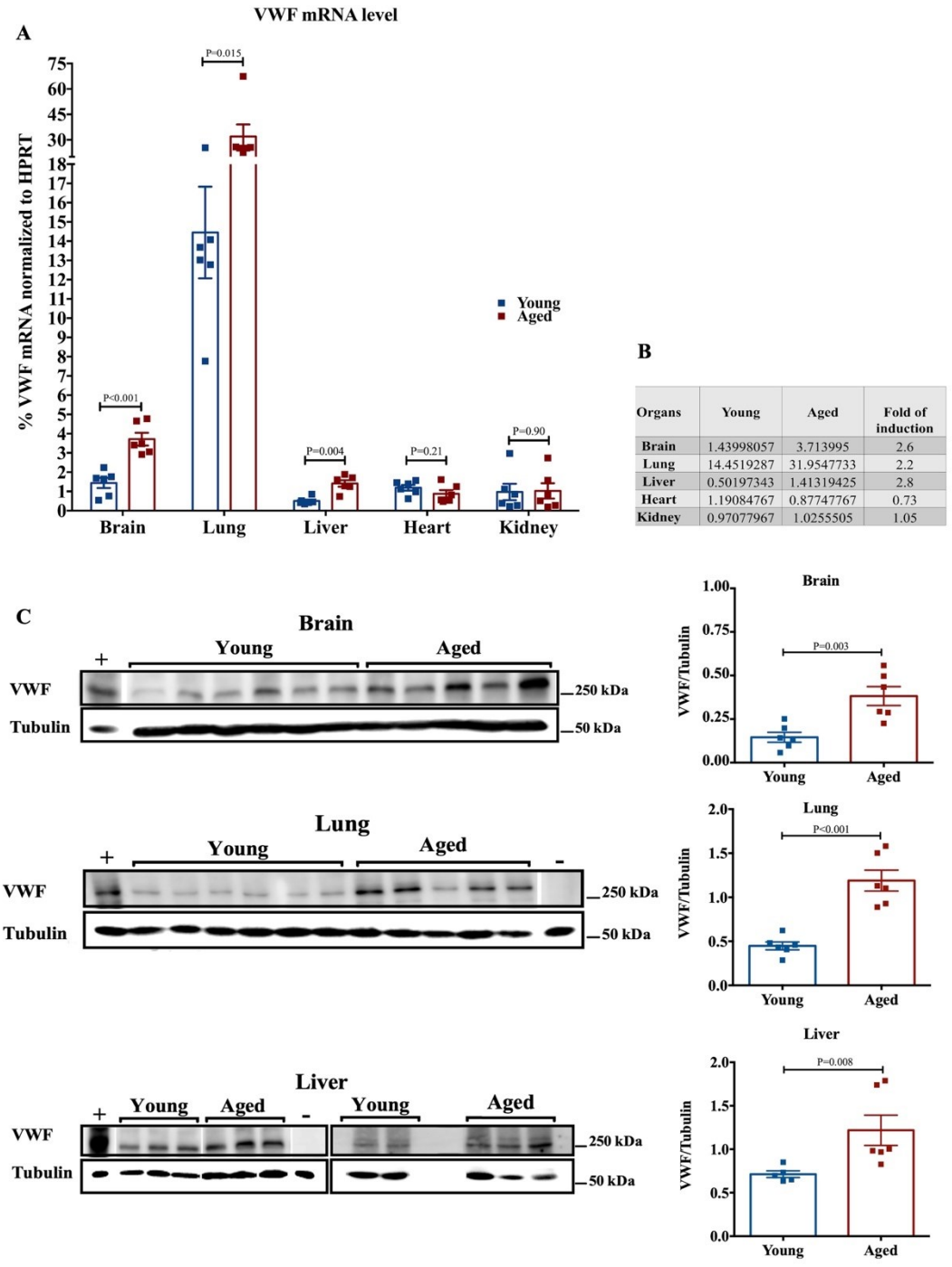


Figure 3.1.1. Analysis of plasma VWF levels, multimer distribution and ADAMTS13 levels.

(A) Concentrations of circulating VWF levels were determined in the plasma of young (3 months) and aged mice (18-19 months). (B) Concentrations of circulating ADAMTS13 levels were determined in the plasma of young and aged mice, as described in "Methods". Data are shown as mean±SEM. (n=6), data are analyzed by the Student *t*-test. (C) VWF multimers analyses of plasmas from 4 young (Lanes 1-4) and 5 aged (lanes 5-9) mice were performed as described in "Methods". HMWM and LMWM represent high and low molecular weight multimers respectively. Region containing HMWM are highlighted in red rectangle.

Our previous analyses regarding the expression of VWF in response to stimuli, specifically hypoxia, demonstrated an organ-specific pattern of VWF transcriptional upregulation [41, 111].

Thus, we proceeded to determine whether age-induced increase in circulating VWF levels reflect increased VWF mRNA levels, and whether the process exhibits organ-specificity. Organs from young and aged mice were used to determine VWF mRNA and protein levels. Since VWF expression is restricted to endothelial cells of the organs, the analyses reflected VWF mRNA and cellular protein levels in endothelial cells. VWF mRNA levels, determined by qRT-PCR, were increased in brains, lungs, and livers of aged mice compared to young, but not in hearts and kidneys (Figure 3.1.2 A-B). VWF cellular protein levels, assessed by Western blot, were also higher in brains, lungs and livers of aged compared to young mice (Figure 3.1.2 C). Collectively these results demonstrated that age-related increase in circulating VWF levels occurred concomitant with organ-specific increases in cellular VWF mRNA and protein.



Organs	Young	Aged	Fold of induction
Brain	1.43998057	3.713995	2.6
Lung	14.4519287	31.9547733	2.2
Liver	0.50197343	1.41319425	2.8
Heart	1.19084767	0.87747767	0.73
Kidney	0.97077967	1.0255505	1.05

Figure 3.1.2. Analysis of VWF levels at mRNA and protein levels in various organs.

(A) Quantitative real time PCR analysis was used to determine the mRNA levels of VWF, normalized to HPRT mRNA, in major organs (liver, lung, brain, heart, and kidney) of young and aged mice. Data are shown as mean±SEM (n=6), analyzed by the Student *t*-test or Mann-Whitney

U test (For the lung, liver, and kidney data sets). (B) Table shows fold induction of VWF mRNA in all aged organs compared to young. (C) Representative Western blot images are shown to detect VWF protein, and tubulin as control, in brain, lung and liver of aged and young mice. The graphs represent densitometry quantification after normalization to tubulin. In Western blots for lung and liver, cellular protein from HEK293 and human umbilical vein endothelial (HUVEC) cells were used as VWF non-expressing negative control (-) and VWF expressing positive control (+) respectively. Data are shown as mean±SEM (n=6 in all data set except in young liver samples n=5), analyzed by the Student *t*-test or Mann-Whitney U test (for the liver data set).

3.1.4.2 Aging is correlated with alterations in vascular tree expression pattern of VWF

VWF expression is mainly restricted to endothelial cells of larger vessels in many organs, specifically liver, lung, and kidney [331-334]. However, our previous investigations into the mechanism of VWF response to hypoxia demonstrated that in addition to increased levels of VWF mRNA, hypoxia treatment (of young mice) resulted in an alteration in the vascular bed expression pattern of VWF [41]. Indeed, immunofluorescence microscopy analyses demonstrated that while VWF was primarily restricted to the large and medium sized vessels of the lung in young mice, hypoxia exposure resulted in VWF expression in a significant number of small vessels as well. The hypoxia-induced alteration in VWF vascular bed expression pattern was highly organ-specific and restricted to the lung, although increased VWF mRNA in all major organs, except kidney, was observed [41]. This observation prompted us to investigate whether aging process may also alter VWF expression pattern in vascular tree of target organs, where expression level is elevated.

Immunofluorescent and confocal microscopy analyses were performed to detect VWF expression and its colocalization with CD31 (an endothelial cell marker). In liver (Figure 3.1.3 A) and lung (Figure 3.1.3 B), as expected large vessels in both young and aged mice expressed VWF**,** however a higher number of small vessels of liver and lung also expressed VWF in aged mice compared to young. In brain, where VWF expression was detectable in microvessels as well as large vessels in both groups of mice, a higher proportion of small vessels expressed VWF in brains of aged mice compared to young (Figure 3.1.3 C). This was demonstrated by quantification analyses of the ratio of CD31 + VWF expressing small vessels to those expressing CD31 without VWF expression (Figure 3.1.3 D**).** VWF expression pattern were unaltered in hearts and kidneys of aged mice compared to young (Figure 3.1.3 E), demonstrating that age does not alter either the levels or the vascular tree expression patterns of VWF in these organs. These results show that an increasing proportion of microvascular endothelial cells in select organs (specifically liver, lung and brain) exhibit VWF expression in response to aging and suggest that this may contribute to a procoagulant shift in phenotypic state of endothelial cells in these vascular beds.

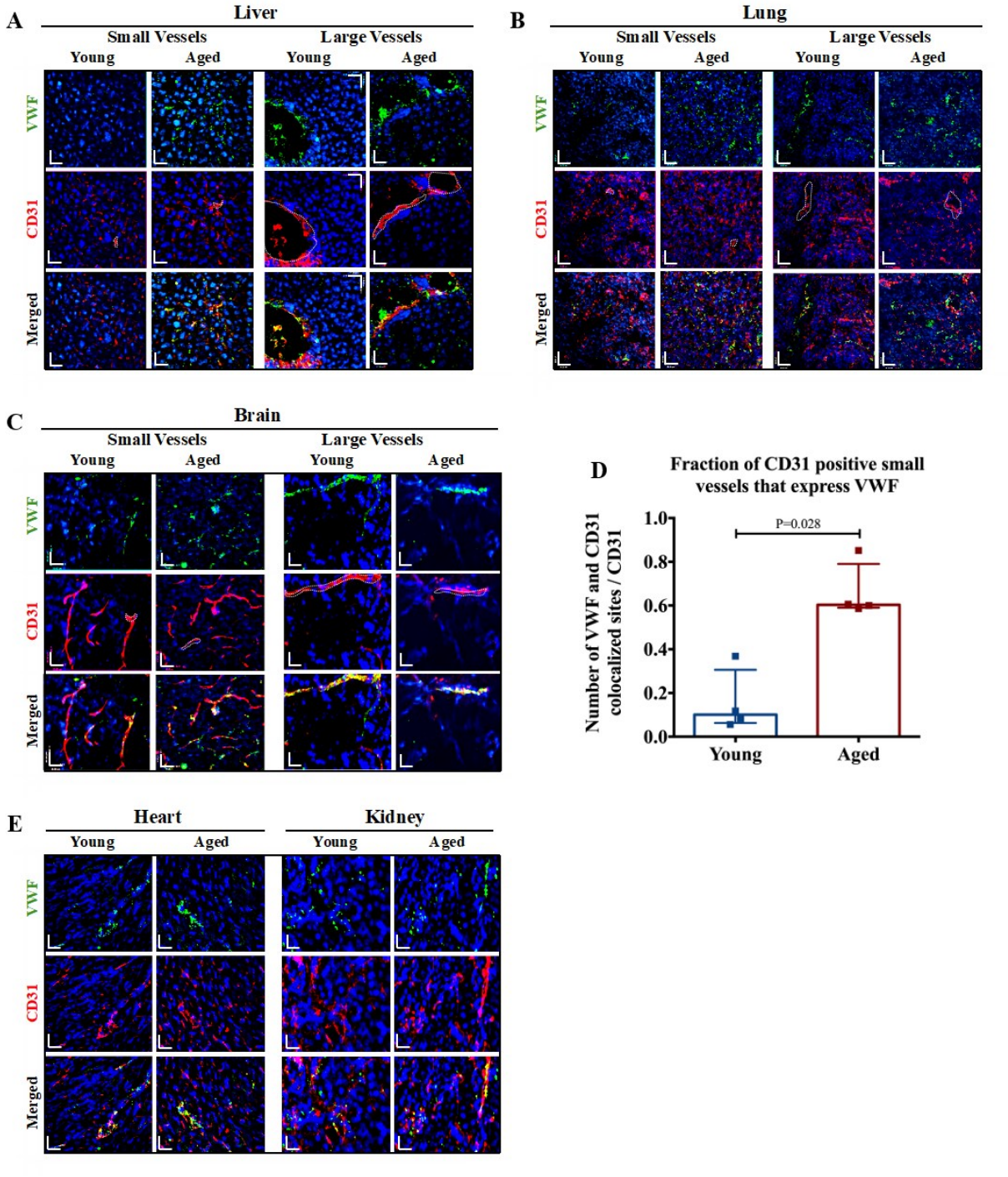


Figure 3.1.3. Immunofluorescent and confocal microscopy analyses of VWF and CD31 in major organs of young and aged mice.

Immunofluorescence co-staining for VWF (green) and CD31 (red) were performed on 5 μm OCT frozen sections of **(A)** liver, **(B)** lung, **(C)** brain, **(E)** heart and kidney of young and aged mice.

Colocalization of VWF with endothelial cells marker (CD31) is detected as yellow. Cell nuclei were visualized using DAPI (blue). Sections of various organs are processed simultaneously under similar conditions, to minimize inter-experimental staining variability. Scale bar is 26 μm. **(D)** Quantitative analysis of the number of micro vessels that express VWF (and CD31) as a fraction of total CD31 expressing microvessels per field of view. 6 field of view were analyzed per animal. Data shown as median±interquartile (n=4), analyzed by the Mann-Whitney U test. Representative vessels are marked with dotted lines in the image.

3.1.4.3 Increased VWF levels in organs of aged mice are concomitant with increased platelets aggregate formation

VWF is a procoagulant protein that mediates platelets aggregation, which is necessary for thrombus formation [68]. To observe the functional consequences of increased VWF expression and to detect whether it is associated with any thrombogenic consequences, we explored the presence of platelet aggregates in the livers, lungs, brains, hearts, and kidneys of aged and young mice. Platelet aggregates, leading to vessels partial or complete occlusion, were observed in small and medium sized vessels of liver and lung, as well as brain microvessels in aged mice (Figure 3.1.4 A). In brains of aged mice, a number of microvessels exhibited partially occlusive platelet aggregates, or trapped platelets adhering to the sides of microvessel walls. Consistent with the lack of alterations in the levels and expression patterns of VWF in hearts and kidneys of aged mice, vasculatures of these organs did not exhibit increased platelet aggregates formation (Figure 3.1.4 B). Immunofluorescence microscopy using anti VWF and anti CD41 specific antibodies demonstrated the colocalization of platelet aggregates with the VWF expressing microvascular endothelial cells in the brain of aged mice (Figure 3.1.4 C).

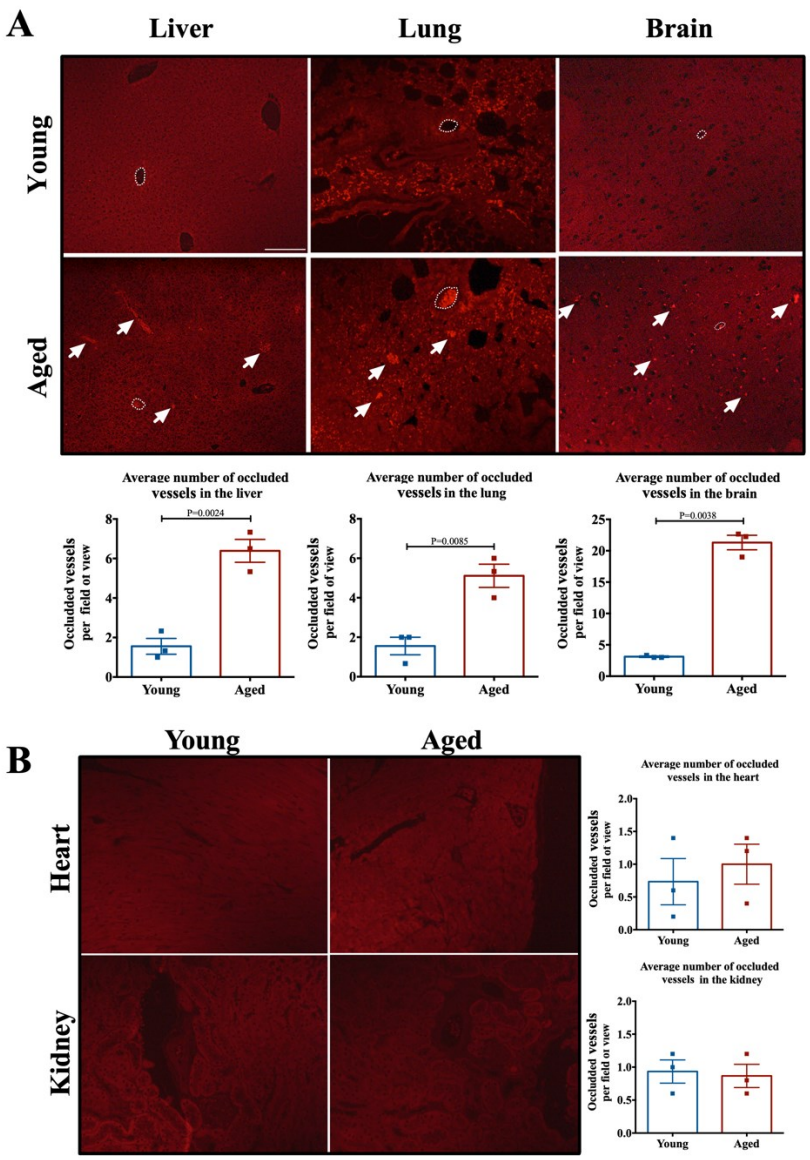

A
Liver
Lung
Brain
Young
Aged
Average number of occluded vessels in the liver
P=0.0024
Occludded vessels per field of view
Young
Aged
Average number of occluded vessels in the lung
P=0.0085
Occludded vessels per field of view
Young
Aged
Average number of occluded vessels in the brain
P=0.0038
Occludded vessels per field of view
Young
Aged
B
Young
Aged
Heart
Kidney
Average number of occluded vessels in the heart
Occludded vessels per field of view
Young
Aged
Average number of occluded vessels in the kidney
Occludded vessels per field of view
Young
Aged
C
Young
Aged
VWF/CD41/DAPI
CD41/DAPI

Figure 3.1.4. Immunofluorescent staining to detect platelet aggregate formation in major organs of young and aged mice.

(A-B) Sections from paraffin embedded organs were subjected to immunofluorescent staining using anti CD41 (a marker of platelets) antibody to detect platelet aggregates as described in methods. White arrows show representative platelets aggregates, while dotted lines show the outline of representative vessels. Graphs represent quantification of platelets aggregates as average number of occluded vessels (regardless of fully or partially occluded) per field of view. 3-5 field of view were analyzed per animal. Data shown as mean±SEM (n=3), analyzed by the Student *t*-test or with Welch correction (for the brain data sets). Scale bar is 50 μm. **(C)** Immunofluorescence co-staining for VWF (green) and CD41 (red) in OCT frozen brain sections of young and aged mice. Arrows show the colocalization sites in yellow, demonstrating the presence of platelets aggregates at the sites of VWF expressing vessels in brains of aged mice. Cell nuclei were visualized using DAPI (blue). Scale bar is 50 μm.

To determine whether we can interfere with the process of platelet aggregates formation, aged mice were treated with low dose acetylsalicylic acid (ASA; aka Aspirin®), an inhibitor of platelet aggregation, prior to euthanasia. ASA reduced platelet aggregate formation in the brain of aged mice compared to the untreated aged control group (Figure 3.1.5 A-B).

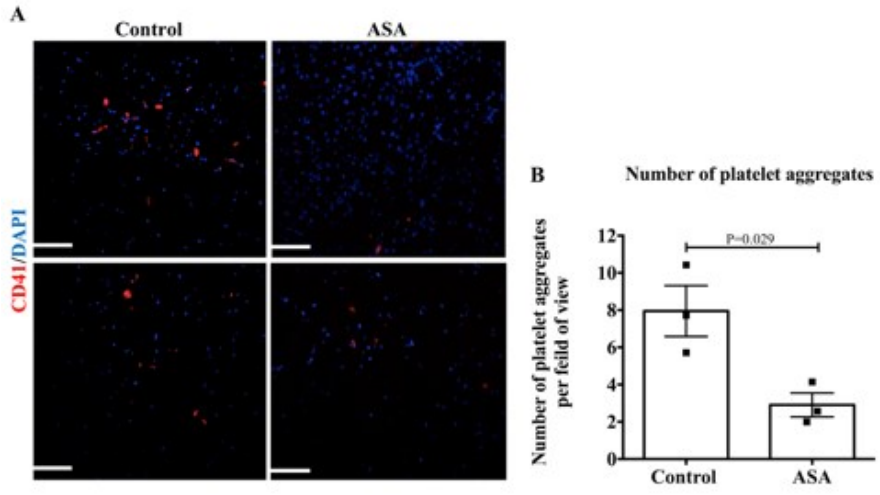


Figure 3.1.5. Detection of platelet aggregate formation in brain vasculature of control and ASA treated aged mice.

(A) OCT frozen brains sections of ASA treated, and control aged mice (for this experiment the aged mice were 24 months old) were subjected to immunofluorescent staining to detect platelet aggregates using CD41 antibody. Cell nuclei were visualized using DAPI. Scale bar is 50 µm. (B) Quantitation of platelet aggregates in control or ASA-treated mice. 10 field of view were analyzed per animal. Data shown as mean±SEM (n=3), analyzed by the Student *t*-test.

These results collectively demonstrate that age-related increase in VWF expression is associated with an increase in partial or fully occlusive platelet aggregates formation in the vessels of lung, liver, and brain; and that compounds with platelets aggregation inhibitory function can interfere with this process.

3.1.4.4 Increased VWF expression was specifically detected in senescent endothelial cell population

Senescent cells accumulate with aging and are associated with age-related tissue and organ malfunction [335]. Thus, we proceeded to determine whether cell senescence could be a participatory mechanism in age-related increase in VWF expression. As an in vitro model of aging, HUVECs were kept in culture for three weeks, without passaging. Cells at week 0 and week 3 were subjected to the qRT-PCR to detect VWF mRNA expression levels. The result indicated an increase in VWF expression in HUVECs maintained for three weeks in culture compared to week 0 (Figure 3.1.6 A). Next, HUVECs maintained in culture at various time points (0, 1, 2 and 3 weeks) were subjected to FACS analysis to assess the expression of the most common marker of cell senescence, senescence associated β-galactosidase activity (SA-β-Gal) [336] by X-Gal staining. Extended time in culture resulted in increasing proportion of cells that expressed SA-β-Gal compared to week 0 (Figure 3.1.6 B). VWF expression also increased in these senescent populations with time in culture (Figure 3.1.6 C). Further, VWF staining intensity was greater in senescent (X-Gal positive) compared to non-senescent (X-Gal negative) cells population (Figure 3.1.6 D).

To directly determine whether cell senescence contributes to age-associated VWF expression in vivo, immunofluorescence co-staining with specific antibodies was performed to detect VWF and SA-β-Gal in the brain's sections of aged and young mice. Colocalization of VWF and SA-β-Gal was detected in a significantly higher proportion of endothelial cells in the brains of aged mice compared to young, which exhibited very few SA-β-Gal + VWF expressing cells

(Figure 3.1.6 E-F). Since we hypothesized that activation of cellular senescence program is a contributing factor towards age-associated stimulation of VWF transcription, we also sought to determine whether VWF expression correlated with the manifestation of the senescence-associated transcription factor p53 [336]. Immunofluorescent co-staining for VWF and p53 demonstrated that a higher proportion of VWF expressing endothelial cells also expressed p53 in brains of aged mice compared to young; an observation that was consistent with that observed for VWF and SA-β-Gal (Figure 3.1.6 G-H). These results collectively demonstrate that the process of cell senescence in endothelial cells is correlated with age-related activation of VWF upregulation and/or *de novo* expression in microvascular endothelial cells of the brain (and most likely other target organs) that otherwise normally may not exhibit VWF expression in vivo.

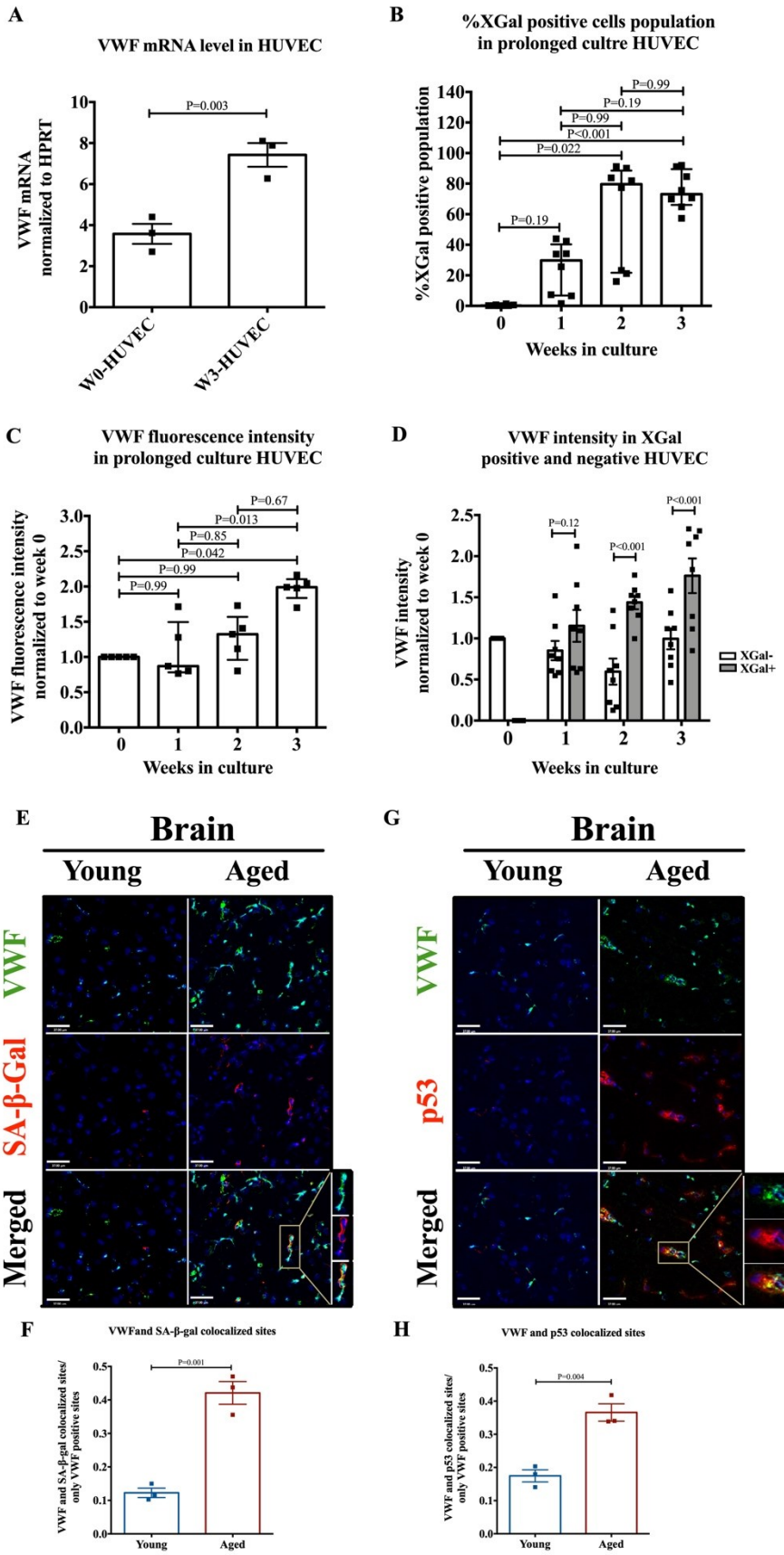

A
VWF mRNA level in HUVEC
VWF mRNA normalized to HPRT
P=0.003
W0-HUVEC
W3-HUVEC
B
%XGal positive cells population in prolonged cultre HUVEC
%XGal positive population
P=0.99
P=0.19
P=0.99
P<0.001
P=0.022
P=0.19
Weeks in culture
C
VWF fluorescence intensity in prolonged culture HUVEC
VWF fluorescence intensity normalized to week 0
P=0.67
P=0.013
P=0.85
P=0.042
P=0.99
P=0.99
Weeks in culture
D
VWF intensity in XGal positive and negative HUVEC
VWF intensity normalized to week 0
P=0.12
P<0.001
P<0.001
XGal-
XGal+
Weeks in culture
E
Brain
Young
Aged
VWF
SA-β-Gal
Merged
G
Brain
Young
Aged
VWF
p53
Merged
F
VWFand SA-β-gal colocalized sites
VWF and SA-β-gal colocalized sites/ only VWF positive sites
P=0.001
Young
Aged
H
VWF and p53 colocalized sites
VWF and p53 colocalized sites/ only VWF positive sites
P=0.004
Young
Aged

Figure 3.1.6. Quantification of senescent cells and VWF expression in human umbilical vein endothelial cells during prolonged culture and in the brain section of aged and young mice.

(A) Quantitative real-time PCR analysis was used to determine the mRNA levels of VWF, normalized to HPRT mRNA, in HUVECs at week 0 compared to week 3. Data shown as mean±SEM (n=3 independent experiments), analyzed by the Student *t*-test. **(B)** Quantification of X-Gal positive population of HUVECs maintained in culture for various times. Data shown as median±interquartile range (n=8 independent experiments), analyzed by Friedman test with Dunn´s post test. **(C)** Quantification of the VWF fluorescence intensity in HUVECs at various time points in culture. Data are shown as median±interquartile (n=5 independent experiments), analyzed by Friedman test with Dunn´s post test. **(D)** Quantification of the VWF fluorescence intensity in X-Gal positive and X-Gal negative populations of HUVECs at various time points. Data are shown as mean±SEM (n=8 independent experiments), analyzed by Two-way ANOVA with Sidak post hoc test. **(E)** Confocal microscopy images of co-immunofluorescence staining of the brain sections of aged and young mice to detect VWF (green) and SA-β-Gal (red). **(F)** Quantitation of the average number of colocalization sites of VWF and SA-β-Gal as a ratio of total VWF expressing microvessels. **(G)** Confocal microscopy images of co-immunofluorescence staining of the brain sections of aged and young mice to detect VWF (green) and p53 (red). **(H)** Quantitation of the average number of colocalization sites of VWF and p53 as a ratio to total VWF expressing microvessels. Cell nuclei were visualized using DAPI. 4-5 field of view were analyzed per animal. Data shown as mean±SEM (n=3), analyzed by the Student *t*-test. Scale bar is 37 μm.

3.1.4.5 Knockdown of p53 reduced the age-associated increase VWF in HUVEC

P53 is known to regulate directly or indirectly the expression of many genes and interact with many partners [337]. The results of immunofluorescent analyses demonstrated an increase in the proportion of p53 and VWF co-expressing endothelial cells in the brain of aged mice, suggesting that p53 may be a contributing factor in regulating age/senescence associated VWF expression. To test this hypothesis, we treated the aged HUVECs with either specific siRNA to knockdown p53 (Sip53) or non-specific siRNA (SiNS) as control and compared VWF expression in siRNA treated and untreated aged cells (W3 maintained in culture for 3 weeks) with that in young (W0) control HUVECs. The successful knockdown of p53 using Sip53 was demonstrated at the protein and RNA levels by Western blot and qRT-PCR analysis of siRNA treated week 0 HUVECs (Figure 3.1.7 A-C). Due to increased cell toxicity when 3 weeks old HUVECs were treated with siRNA, sufficient cells were not obtained for Western blot analysis to assess p53 protein; however, we confirmed p53 knockdown in these cells at the RNA level by qRT-PCR (Figure 3.1.7 D). Cells were then subjected to qRT-PCR analysis to determine VWF mRNA levels in the p53 knocked down (Sip53) compared to SiNS and untreated aged HUVECs (W3) as well as untreated young HUVECs (W0). While the VWF levels in untreated and SiNS treated W3 HUVECs were higher than that in W0 HUVECs, the VWF levels in Sip53 treated W3 HUVECs were similar to that observed in W0 HUVECs (Figure 3.1.7 E). These results demonstrated that the age-induced VWF upregulation was abolished in response to p53 knockdown, supporting the participation of p53 in regulating age/senescence-associated increase in VWF expression.

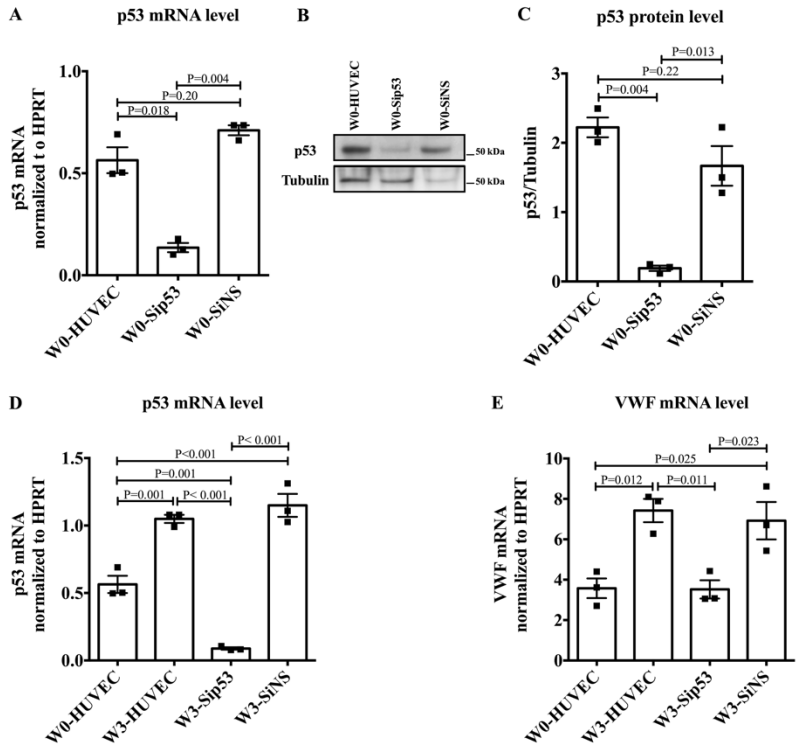


Figure 3.1.7. Knockdown of p53 using siRNA in HUVECs.

RNA and protein were prepared from HUVECs at week 0 (W0) or week 3 (W3) that were untreated, treated with p53 specific siRNA (Sip53), or with nonspecific siRNA (SiNS). (A) RNA from W0 HUVECs were subjected to qRT-PCR analysis to detect p53 mRNA levels. (B) Proteins prepared from W0 HUVECs were subjected to Western blot analyses to detect p53 and Tubulin. (C) Densitometry quantification after normalization to tubulin. (D) RNA from W3 HUVECs were subjected to qRT-PCR analysis to detect p53 mRNA. (E) RNA from W3 HUVECs and untreated W0 HUVECs were subjected to qRT-PCR analysis to detect VWF mRNA levels. Data shown as mean±SEM (n=3 independent experiments), analyzed by one-way ANOVA with Tukey post hoc test.

3.1.5 Discussion

A variety of stimuli are known to mediate VWF release and generally once the stimulus is terminated, the release of VWF returns to the basal level [338]. The control and regulation of VWF levels, in combination with processes that participate in its clearance, allows for generation of

platelet plugs when required, and their clearance once the repair process is completed and initiating stimulus is terminated [339, 340]. However, if VWF expression is altered in response to a sustained stimulus, it may remain upregulated, and cause a pathological state of platelet aggregates formation, which could hinder blood flow through the vasculature [325]. It has been estimated that VWF levels increase with aging by a rate of 1% per year in healthy human population [320]. Since aging is associated with increased thrombogenicity and its pathological consequences [341], we explored whether aging may function as a sustained stimulus for VWF upregulation, with functional consequences contributing to increased thrombogenicity.

In this study, we used male mice as a model for aging and demonstrated that plasma levels of VWF were significantly increased in aged mice compared to young. Moreover, VWF multimer distribution pattern analysis demonstrated an increased plasma level of HMWM VWF in aged compared to young mice. This suggests that age-induced elevated levels of circulating VWF include larger multimers that are biologically active and functional, and thus potentially associated with higher risk of cardiovascular and thrombotic diseases. Increased VWF levels in circulation may originate from the degranulation of the storage organelles with or without alterations in VWF transcriptional regulation [47]. However, VWF transcripts, as well as cellular proteins, were elevated, in select organs of aged mice compared to young, suggesting an effect on the VWF transcriptional regulation. We hypothesize that elevated circulating levels of HMWM is reflective of increased transcriptional/translational activity of VWF gene that culminates in large multimers generation and cellular storage. Since VWF release from endothelial cells can occur through either constitutive, or regulated and basal release from WPB [34], the age-associated elevated HMWM may reflect basal release that occurs due to random degranulation of WPB. In response to injury

or stimuli, such as inflammation, the regulated release may further elevate circulating HMWM VWF levels in aged organism and consequently exacerbating the thrombogenicity. The results also demonstrated that aging not only alters potentially transcriptional activities of the VWF gene, but it does so in an organ-specific manner. Although we cannot rule out the possibility that increased VWF mRNA may reflect increased mRNA stability, considering that VWF mRNA is generally stable (half-life > 8 hours) [4], the elevated levels likely reflect increased transcriptional activities of the VWF gene. Thus, suggesting that aging functions as a stimulus that targets VWF gene transcription. Additional support for this hypothesis is based on the observation that ageing not only upregulated VWF levels in an organ-specific manner (since it was detected in liver, lung and brain but not kidney or heart) but also it resulted in de novo activation of VWF expression in microvascular endothelial cells of target organs (liver, lung and brain) of aged mice.

Although all cultured endothelial cells, including microvascular endothelial cells, exhibit VWF expression, endothelial cells in vivo present a heterogeneous pattern of VWF expression [342]. Studies performed to evaluate VWF expression pattern are mostly based on the analyses of relatively young rodents, which demonstrated that VWF expression is patchy and relatively rare or non-detectable in microvasculature of many organs, while uniformly detected in large vessels [342, 343]. Specifically, in liver, VWF is only detected in large vessels and not in small vessels or capillaries [331, 343]. In lung, capillaries and microvessels either do not exhibit VWF expression, or exhibit a mosaic pattern, while large vessels of more than 40 microns uniformly express VWF [331-333]. In the brain, microvessels as well as large vessels exhibit VWF expression, but the expression in microvessels is mosaic and a significant proportion do not express VWF [343]. Our analyses demonstrate that while VWF expression in liver, lung and brain of young mice exhibited

an expression pattern that was consistent with previous studies, an increasing number of microvessels in these organs exhibited VWF expression in aged mice. In the brain, we also observed an increase in proportion of microvessels that express VWF in aged compared to young mice. These data strongly suggest that while aging may result in upregulation of VWF in endothelial cells that normally support its expression, it also results in de novo transcriptional activation of the VWF gene in microvascular endothelial cells of liver, lung, and brain, that otherwise in the young organs, keep VWF gene expression supressed.

The increased VWF expression in target organs of aged mice was coincident with increased platelet aggregates formation, suggesting a functional consequence of age-associated increase in VWF expression. Based on these observations we propose that endothelial cells of distinct organs respond differentially to aging with regard to regulation of VWF gene expression, leading to potential functional consequences associated with thrombogenicity. The observation that increased platelet aggregate formation was detected in the vasculature of organs that exhibited age associated increased VWF (liver, lung, and brain), but not in organs that did not exhibit this phenomenon (heart and kidney), indicates that potential functional consequences of altered VWF expression are manifested locally. In liver and lung, the presence of platelet aggregates in large/medium size vessels (as well as microvessels) supports the hypothesis that in addition to *de novo* activation of VWF in microvascular endothelial cells, endothelial cells of larger vessels that normally express VWF in young, may also increase their VWF expression leading to potential functional consequences. Alternatively, the observed platelet aggregates in larger vessels may be correlated to increased circulating VWF levels that initiates from de novo activation of expression in microvascular endothelial cells and entering local circulation in these organs.

Presence of platelet aggregates is not necessarily correlated to thrombus formation, since many such aggregates may form without fibrin deposition for stabilization, and then resolve through the action of various enzymes, including VWF cleaving enzymes [344]. This is consistent with our observation regarding the results of aspirin treatment, an inhibitor of platelet aggregate formation, which lead to a significant reduction in the number of platelet aggregates detected in the brain. Nevertheless, dynamics of increased rate of platelet aggregate formation and resolution, may present conditions in which, the target organ may not be optimally perfused at all times, leading to organ dysfunction over time. This maybe specifically relevant to the brain since we observed a significant number of platelet aggregates in numerous microvessels of the brain that fully or partially occluded the vessels. Suboptimal brain perfusion may be a contributing factor to age-associated decline in brain function and development of states such as vascular cognitive impairment [345]. To investigate the underlying mechanisms of age-related VWF upregulation, we used both in vitro and in vivo models. VWF expression in HUVECs increased with the length of time in culture and was most pronounced in cells that exhibited markers of senescence. Confocal immunofluorescence analysis of the brain sections of aged and young mice also demonstrated that a higher proportion of VWF expressing endothelial cells exhibited senescence markers SA-β-galactosidase and transcription factor p53 in the brains of aged mice compared to young. This was especially prominent when SA-β-galactosidase was used, since p53 may also be detectable in actively dividing cells (which are expected to be few among vascular endothelial cells in adults under normal circumstances)[337] . However, p53 is not exclusively a senescence marker and is transiently expressed in metabolically active cells during cell division, hence its presence in many cancers cell types [346]. Due to the transitory nature of its expression, p53 is not usually detected under physiological condition in adult organs [337, 346]. Therefore, its detection is a relevant

indication of cell senescence, which in combination with SA-β-galactosidase staining would provide a reliable strategy to detect senescent cells [336, 347].

The contribution of cell-senescence program to VWF activation is supported by recent reports of Mojiri et al [348], which demonstrated elevated levels of VWF expression in isolated aortic endothelial cells from Hutchinson-Gilford progeria syndrome (HGPS) mice (a mouse model of rare genetic disease that leads to accelerated senescence) compared to wild type control. These mice exhibit accelerated aging and an increase in proportion of endothelial cells that undergo senescence. This is also consistent with a report from Luo *et al.*, demonstrating VWF upregulation in hepatic sinusoidal endothelial cells (HSECs) that undergo cell senescence [349]. In addition to increased VWF expression in senescent endothelial cells themselves, the phenomenon of senescence-associated secretory phenotype that is associated with the release of pro-inflammatory molecules by these cells[348] may also contribute to release/production of VWF from neighboring non-senescent endothelial cells, thus further amplifying the process of VWF upregulation in circulation.

We propose that age-related transcriptional alterations in VWF gene expression is mediated through processes that are invoked as a result of cellular senescent and hypothesize that activation of p53 transcription factor participate in this process. In combination with detection of p53 in VWF expressing endothelial cells in vivo, the inhibition of age-associated increase in VWF in cultured endothelial cells as a result of p53 knock down, strongly supports this hypothesis.

A limitation in this study was using only male mice and we cannot rule out the possibility that some or all of the observed results may be dependent on biological sex. We need to consider sex differences and perform similar experiments in female mice in the future studies. In addition,

we used 3 month old mice as a representative of our young group. It should be noted that this age group in mice would be equivalent to human age ranging from 20-30 years, which represents mature adults. In the current study, we chose to name our 3 month old cohort as “young”. This definition needs to be distinguished from the term young in reference to human, which indicates pre-pubescence.

Collectively, the current study provides novel information regarding the processes that contribute to age-associated increase in circulating VWF. It demonstrates that the aging process alters phenotypic profile of microvascular endothelial cells in select organs, leading to increased expression of procoagulant VWF protein; and that the process is coincident with increased platelets aggregate formation and reversible vascular occlusion. Platelet aggregates formation may be a reversible process, but could hamper blood flow in microvasculature, and thus impair target organs’ optimal functions, for instance contributing to development of vascular cognitive impairment in the brain. Demonstration of cell senescence, and a role for p53, as contributing factors to this process provides opportunities for development of anti-senescent therapies as a potential approach towards combating some age related occlusive microvascular diseases, which may lead to target organs’ suboptimal function.

3.1.6 Acknowledgments

None

3.1.7 Sources of Funding

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3.1.8 Disclosures

None

3.1.9 Graphical Abstract

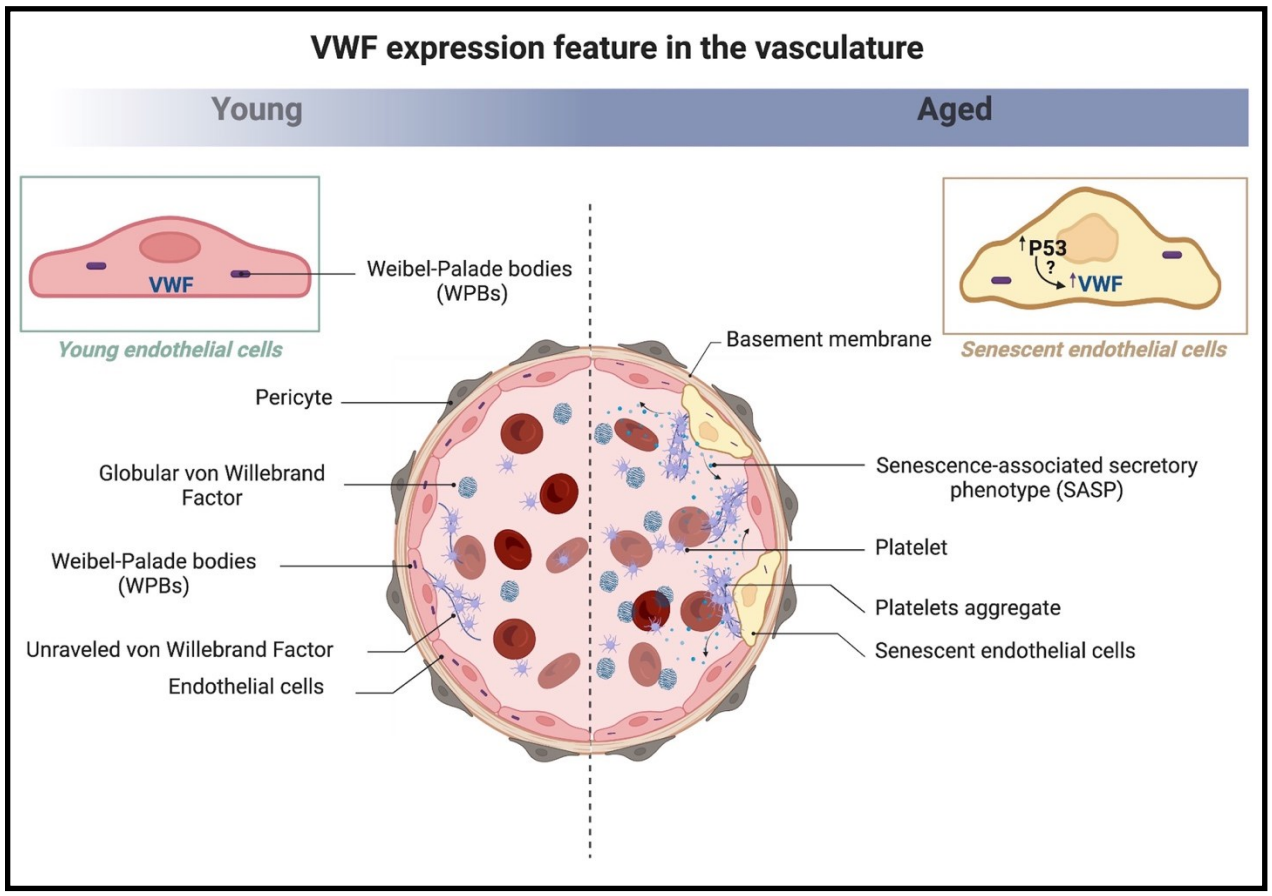


Figure 3.1.8. Graphical Abstract.
Aging induces changes in von Willebrand factor levels and distribution across organs vasculatures. This process is mediated through cell-senescence associated p53. The figure created by Bio Render.

3.1.10 Supplemental material

Table 3.1-1. Primer's gen ID/sequences for quantitative real-time PCR (qRT-PCR)

Gene	Primer name	Source	GeneGlobe ID/Sequences
Mouse VWF	Mm_Vwf_1_SG QuantiTect Primer Assay	QIAGEN	QT00116795
Mouse HPRT	Mm_Hprt_1_SG QuantiTect Primer Assay	QIAGEN	QT00166768
Human VWF	Hs_VWF_1_SG QuantiTect Primer Assay	QIAGEN	QT00051975
Human HPRT	Hs_HPRT1_1_SG QuantiTect Primer Assay	QIAGEN	QT00059066
Human p53	Human p53	IDT	Reverse: 5′-TGGATGGTGGTACAGTCAGAGC-3′ Forward: 5′-CCTCAGCATCTTATCCGAGTGG-3′

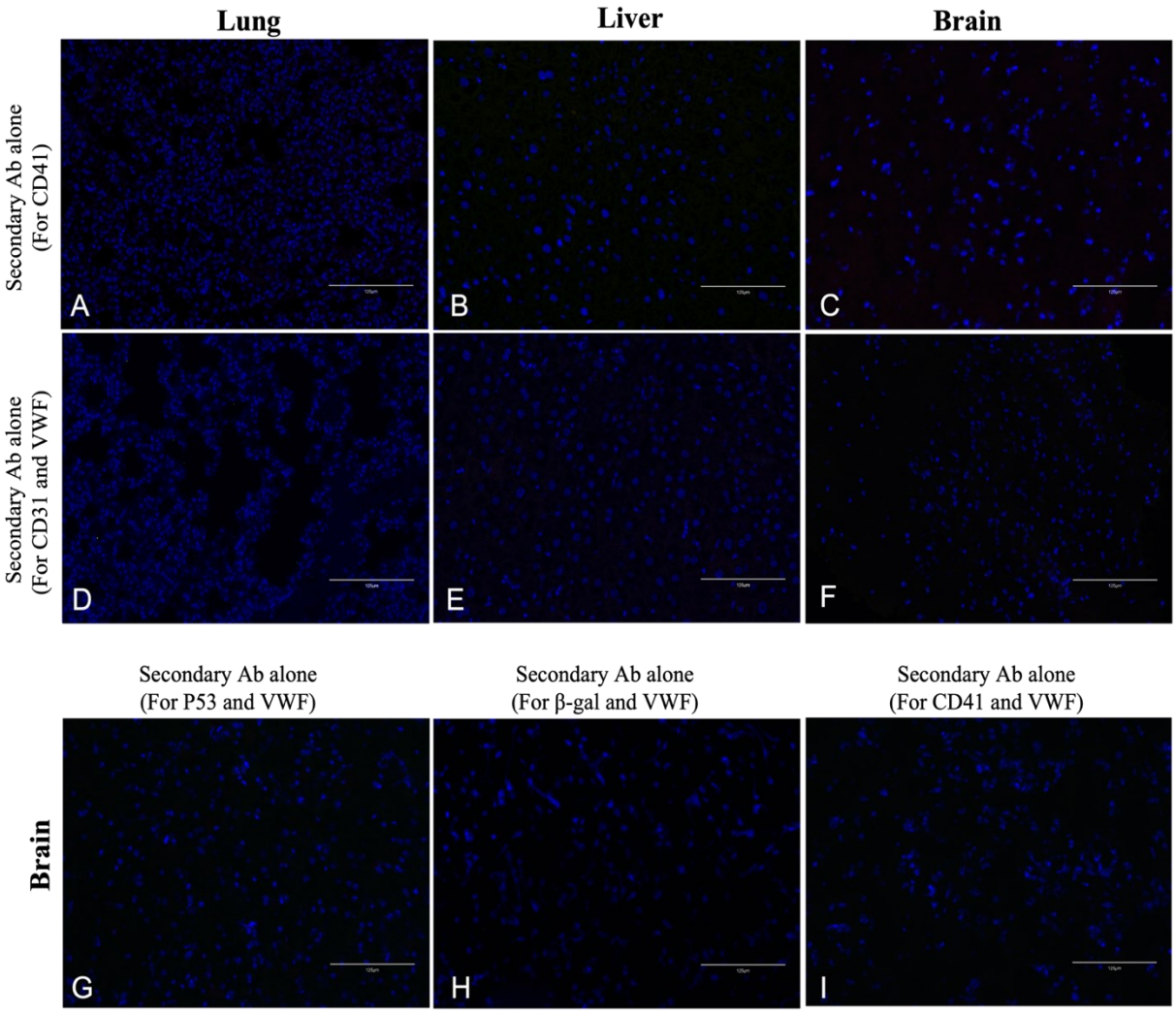
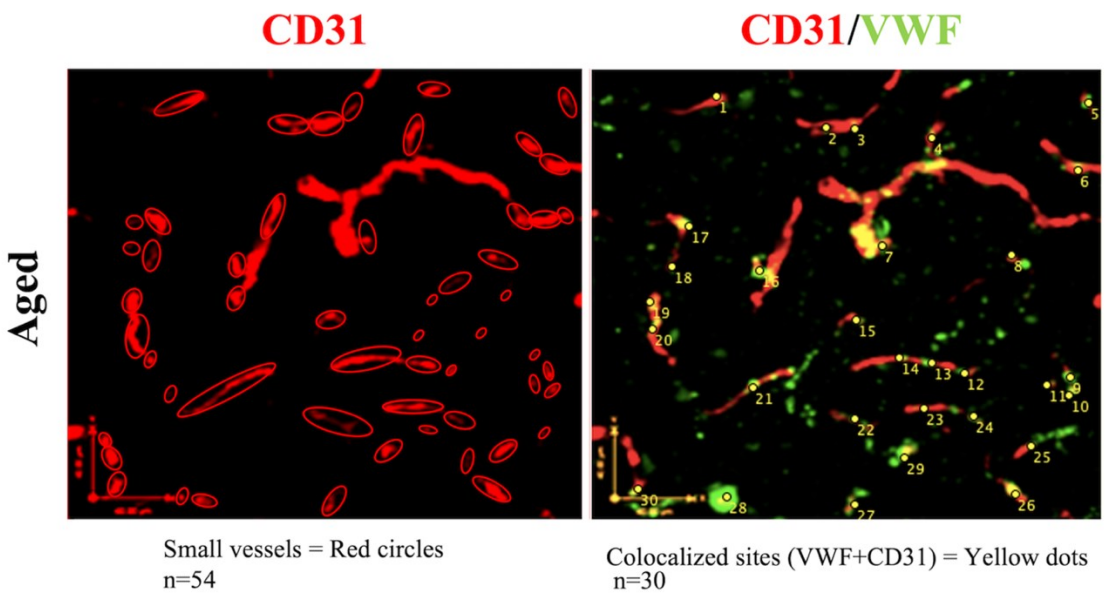


Figure 3.1.9. Negative controls of immunofluorescence images.

(A-I) Sections from different organs were incubated with the secondary antibody alone as negative controls. (A-C) Alexa Fluor 568 secondary antibody was used. (D-F) Alexa Fluor 488 and Alexa fluor 594 secondary antibodies were used. (G-I) Brain sections were incubated with Alexa Fluor 488 and Alexa Fluor 594 or Alexa Fluor 568 secondary antibodies only as negative controls. Cell nuclei were visualized using DAPI (blue). Scale bar is 125 μm.



$$\frac{\text{CD31+VWF}}{\text{CD31}} = \text{Fraction of small vessels that espress VWF}$$

$$\frac{30}{54} = 0.55$$

Figure 3.1.10. The Quantification of small vessels that express VWF in the brain sections

Vessels were identified by CD31 expression and those considered small vessels and quantified are marked by red circles. Colocalized sites of VWF and CD31 are shown by yellow dots and quantified. For example, in the current image of an aged brain 54 red circles were counted as small vessels and 30 yellow dots were counted as colocalized sites. Then the ratio of the number of colocalized sites (Yellow dots) over total small vessels (red circles) were calculated to obtain the ratio of the small vessels that express VWF.

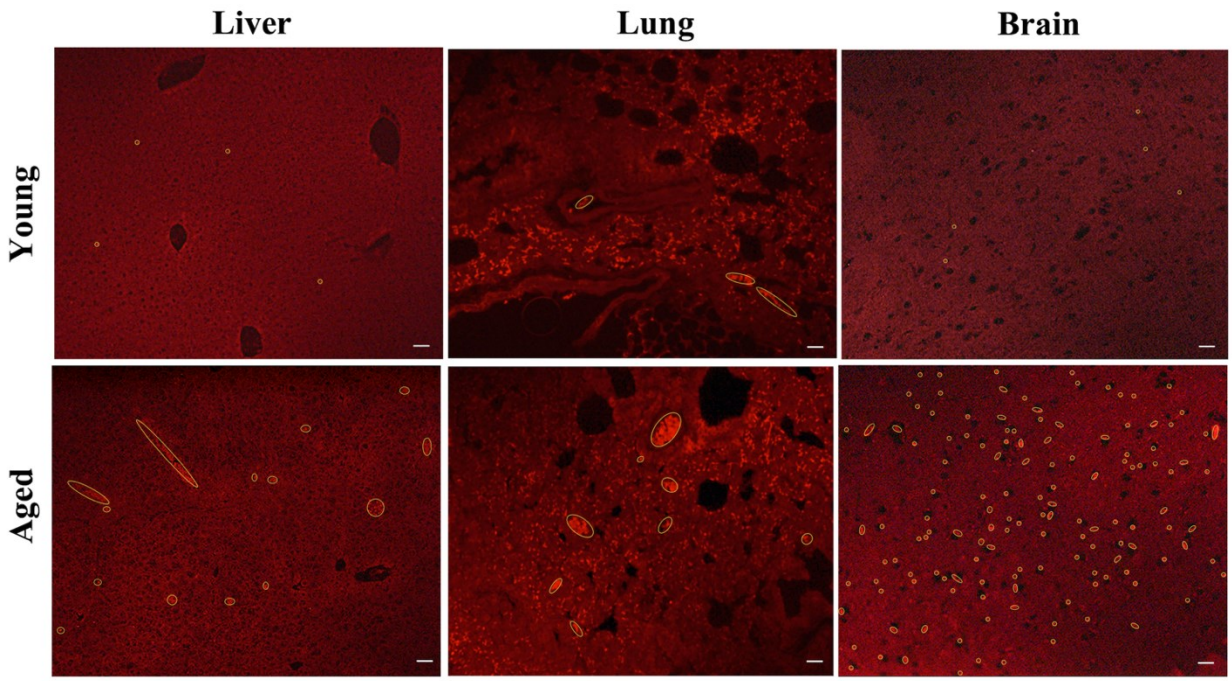


Figure 3.1.11 Identification of platelet aggregates in young and aged organs.

Platelet aggregates that were counted are shown by yellow circles in various organs sections in young and aged mice.

3.1.11 Authors' contribution

PA, RY, AM, and DB performed experiments, data collection and data analysis. SB provided aged and young mice and performed aspirin treatment experiments. JN and JL provided resources, supervision, and intellectual input. NJ, SB, JN, and JL contributed to design of research. PA and NJ wrote the manuscript. NJ provided resources and overall supervision.

3.1.12 Major resources table

Table 3.1-2. Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex
Mice	Charles River	C57BL/6	Male

3.1.12.1 Antibodies

Table 3.1-3. List of antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Persistent ID / URL
Rabbit anti-Von Willebrand Factor antibody	Abcam	ab9378	1:500 (concentration not available)	https://www.abcam.com/von-willebrand-factor-antibody-ab9378.html
Goat anti-Rabbit IgG H&L (HRP)	Abcam	ab97051	(0.1 µg/ml)	https://www.abcam.com/goat-rabbit-igg-hl-hrp-ab97051.html
Mouse anti-α-Tubulin antibody, clone DM1A	Millipore Sigma	05-829	(0.2 µg/ml)	https://www.emdmillipore.com/CA/en/product/Anti-Tubulin-Antibody-clone-DM1A,MM_NF-05-829
Goat anti-mouse IgG antibody, HRP conjugate	Millipore Sigma	12-349	(0.05 µg/ml)	https://www.emdmillipore.com/CA/en/product/Goat-Anti-Mouse-IgG-Antibody-HRP-conjugate,MM_NF-12-349
Sheep anti-Von Willebrand Factor antibody	Abcam	ab11713	1:100 (concentration not available)	https://www.abcam.com/von-willebrand-factor-antibody-ab11713.html
Mouse anti p53 antibody (DO-1)	Santa Cruz	sc-126	(0.4 µg/ml)	https://www.scbt.com/p/p53-antibody-do-1

Rabbit anti beta galactosidase antibody	Orbyte	orb100205	(1 ug/ml)	https://www.biorbyt.com/beta-galactosidase-antibody-orb100205.html
Rabbit anti-CD31 antibody	Abcam	ab28364	1:50 (concentration not available)	https://www.abcam.com/cd31-antibody-ab28364.html
Rat anti- CD41 antibody	Abcam	ab33661	(2.5 µg/ml)	https://www.abcam.com/cd41-antibody-mwreg30-ab33661.html
Goat anti-Rat IgG (H+L) Cross- Alexa Fluor 568	Thermo Fisher scientific	A-11077	(1 µg/ml)	https://www.thermofisher.com/antibody/product/Goat-anti-Rat-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11077
Goat anti-Rabbit IgG (H+L) Highly Cross- Alexa Fluor 594	Thermo Fisher scientific	A-11037	(1 µg/ml)	https://www.thermofisher.com/antibody/product/Goat-anti-Rabbit-IgG-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11037
Donkey anti-Sheep IgG (H+L) Cross-Alexa Fluor 488	Thermo Fisher scientific	A-11015	(1 µg/ml)	https://www.thermofisher.com/antibody/product/Donkey-anti-Sheep-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11015
Donkey anti-Mouse IgG (H+L) Highly Cross- Alexa Fluor 594	Thermo Fisher scientific	A-21203	(1 µg/ml)	https://www.thermofisher.com/antibody/product/Donkey-anti-Mouse-IgG-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-21203
Donkey anti-Sheep IgG (H+L) Cross-Alexa Fluor 647	Thermo Fisher scientific	A-21448	(2.5 µg/ml)	https://www.thermofisher.com/antibody/product/Donkey-anti-Sheep-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-21448

3.1.12.2 Cultured cells

Table 3.1-4. Cells (in vitro studies)

Name	Vendor or Source
Human umbilical vein endothelial cell (HUVEC)	ATCC
Human embryonic kidney 293 cells (HEK293)	ATCC

3.1.12.3 Other materials and methods

Table 3.1-5. List of materials

Description	Source / Repository	Persistent ID / URL
Mouse VWF ELISA kit	Elabscience (E-EL-M1247)	https://www.elabscience.com/p-mouse_vwf_von_willebrand_factor_elisa_kit-21096.html
Mouse ADAMTS13 ELISA kit	Abbexa (Abx514433)	https://www.abbexa.com/mouse-adamts13-elisa-kit
SYBR Green PCR Master Mix	Applied Biosystems (4309155)	https://www.thermofisher.com/order/catalog/product/4309155
Dako antibody diluent	Agilent	https://www.agilent.com/store/productDetail.jsp?catalogId=S080983-2
Gelatin from cold water fish skin	Sigma	https://www.sigmaaldrich.com/CA/en/product/sigma/g7041
RIPA Lysis Buffer System	Santa Cruz	https://www.scbt.com/p/ripa-lysis-buffer-system
Quantitect Reverse Transcription Kit	QIAGEN	https://www.qiagen.com/us/products/discovery-and-translational-research/pcr-qpcr-dpcr/real-time-pcr-enzymes-and-kits/reverse-transcription-cdna-synthesis-qpcr/quantitect-reverse-transcription-kit
eBioscience Intracellular Fixation & Permeabilization Buffer Set	Invitrogen	https://www.thermofisher.com/order/catalog/product/88-8824-00
Western Blocker Solution	Millipore Sigma	https://www.sigmaaldrich.com/CA/en/product/sigma/w0138

3.1.12.4 Study Design

Table 3.1-6. Study design

Groups	Sex	Age	Number (prior to experiment)	Number (after termination)	Littermates (Yes/No)	Other description
Group 1 (Young)	Male	3 months	n=6	NA	NA	Six mice were euthanized for each group. They included different sets of littermates.
Group 2 (Aged)	Male	18-19 and 24 months	n=6	NA	NA	Six mice were euthanized for each group. They included different sets of littermates.

3.1.12.5 Inclusion criteria:

C57BL/6 male mice at the age of 3 months (young) and at the 18-19 and 24 months (aged) groups.

3.1.12.6 Exclusion criteria:

Female mice were excluded. We have used only male mice in our study

3.1.13 Highlights:

- Circulating biologically active VWF levels in mice are increased in response to aging. VWF mRNA and protein are upregulated in aged mice in an organ-specific manner.
- The increasing proportion of microvessels exhibit VWF expression in organs of aged mice.
- Increased VWF expression is accompanied with increased platelet aggregate formation in aged mice.
- Senescent markers were detected in aged endothelial cells that exhibited increased VWF expression.
- P53 knockdown abolished the aged- induced VWF upregulation in endothelial cells.

4 Chapter 4

4.1 Expanding the aging study

4.1.1 In vitro model of endothelial cells aging: In three-week-old HUVECs VWF large multimers showed an increase in both media and cell lysates

As mentioned in Chapter 3 (Figure 3.1.1) we previously demonstrated that the plasma of aged mice compared to young exhibited not only elevated levels of VWF antigen but also an increase in high molecular weight VWF multimers, as detected by multimer analysis.

As part of further investigation, we conducted multimerization assays on the media and cell lysate of three-week-old HUVEC (W3) in our *in vitro* model and compared them to the control [(W0) HUVECs maintained for 72 hours in culture].

The results demonstrated that VWF large multimers were significantly increased in cell lysates and conditioned media in W3 compared to W0 HUVECs (Figure 4.1.1)

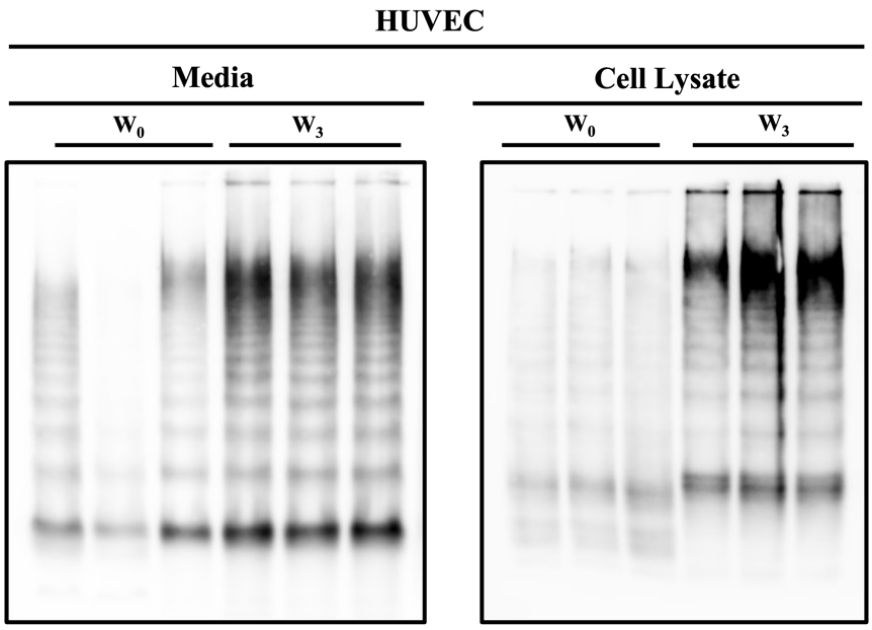


Figure 4.1.1. Multimeric analysis of von Willebrand factor.
Multimeric patterns of VWF were analysed in media and cell lysate of human umbilical vein endothelial cells (HUVECs) which were kept in culture for three days (W0) and three weeks (W3).

4.1.2 VWF large multimers are increased in aged organs

Next, to investigate the multimeric composition of VWF in aged organs, lung and brain tissue lysates from young and aged mice were assessed by multimer analysis. The results demonstrated a significant increase in the high molecular weight multimers of the VWF in the organs (lung and brain) of aged compared to young mice. These *in vitro* and *in vivo* results collectively demonstrate that age/senescence associated VWF upregulation leads to significantly increased generation and intracellular storage of functional high molecular weight multimers of VWF, which may contribute to the observed increased local organ-specific platelet aggregates formation as demonstrated in previous Chapter and also shown here (Figure 4.1.2 A-B).

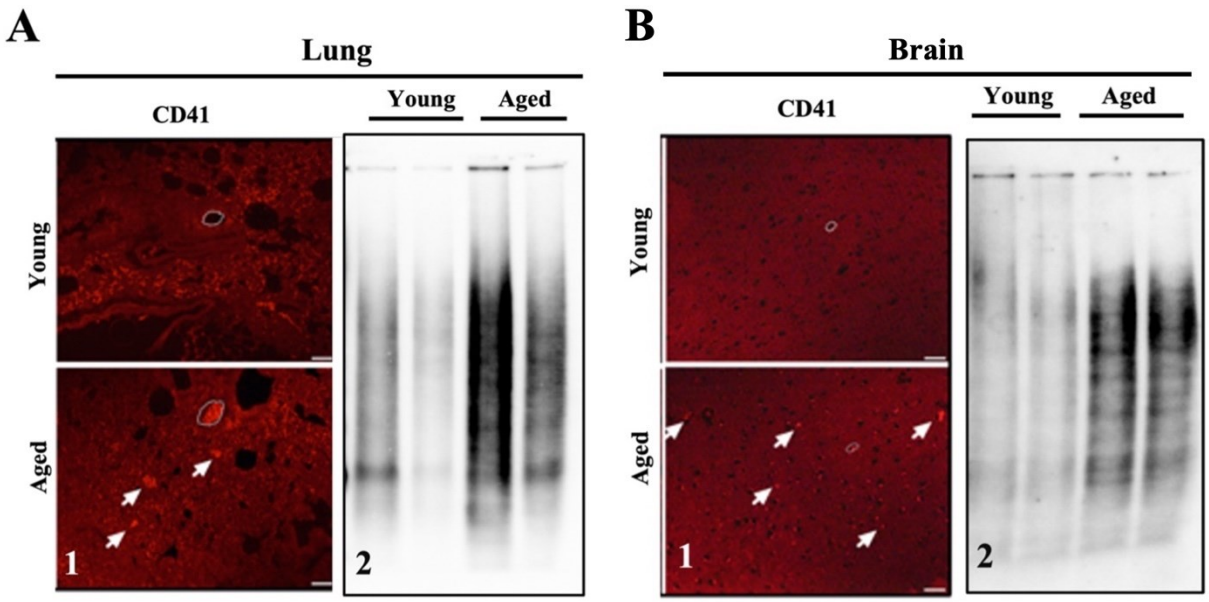


Figure 4.1.2. Analyses of intracellular multimeric composition of the VWF in the lung and brain tissue lysates.

(A-1 and B-1) Formation of platelet aggregates in the lung and brain sections of aged mice, as revealed by immunofluorescent (IF) analyses using anti-CD41 (a platelet-specific marker) antibody, (representative indicated by arrows). (A-2 and B-2) Evaluation of the intracellular multimeric structure of VWF in organs (lung and brain) of young and aged mice.

4.1.3 Von Willebrand Factor expression patterns in progeria mice, an animal model for accelerated aging

Progeria or Hutchinson-Gilford progeria syndrome (HGPS) is rare genetic condition in human that causes accelerated aging in children [350, 351]. It is characterized by symptoms such as hardening and tightening of skin, problems with growth and development of bones, joint stiffness, atherosclerosis, and a range of cardiovascular complications [352, 353]. Unfortunately, patients with progeria would die at the mean age of 13 mainly due to stroke and other cardiovascular disease.

The underlying cause of progeria is a mutation in LMNA gene, which codes for a lamin A protein. Lamin A is the scaffolding that stabilized the nucleus [354] . However, abnormal mutated Lamin A, called progerin, makes the nucleus unstable and lead to the premature aging process observed in progeria. Evidence indicates that progerin, which is normally present at reduced levels in healthy people, gradually builds up in the coronary arteries as people get aged. This finding supports the idea that progerin contributes to the risk of cardiovascular disease associated with aging [355]. Many researchers propose that the abnormal aging observed in HGPS patients results from the accumulation of cellular damage, ultimately affecting the normal cellular function [356].
The effects of progerin extend to various cellular processes, including gene expression, heterochromatin arrangement, DNA repair, and nuclear structure [357].

While the majority of studies focused on loss of vascular smooth muscle cell (VSMC) in progeria patient, which is a typical characteristic of progeria, previously Xu et al, showed that there is a great dysfunction in HGPS-ECs compared to HGPS-VSMCs [358]. This research showed since endothelium is a key player in vascular hemostasis, there is a necessity to investigate the role of endothelial in progeria as well. The Study indicated that progerin induces a senescence phenotype in both vascular smooth muscle cells and endothelial cells, with a more pronounced effect observed in the latter [358].

In collaboration with Dr. Mojiri, we accessed tissue samples from wild-type (WT) and progeria mice, which provided a unique opportunity to extend our study. Therefore, to investigate whether a pathological accelerated aging condition also results in increased and altered von

Willebrand factor expression patterns, we conducted a co-staining immunofluorescence analysis on brain sections from WT and progeria mice.

The result demonstrated that in the brains of progeria mice, a higher proportion of small vessels expressed VWF compared to WT littermate (Figure 4.1.3 A-B)**.** This was quantified (as described for aged and young mice in Chapter 3 (Figure 3.1.3) by analyzing the ratio of vessels expressing both CD31 and VWF to vessels expressing CD31 alone.

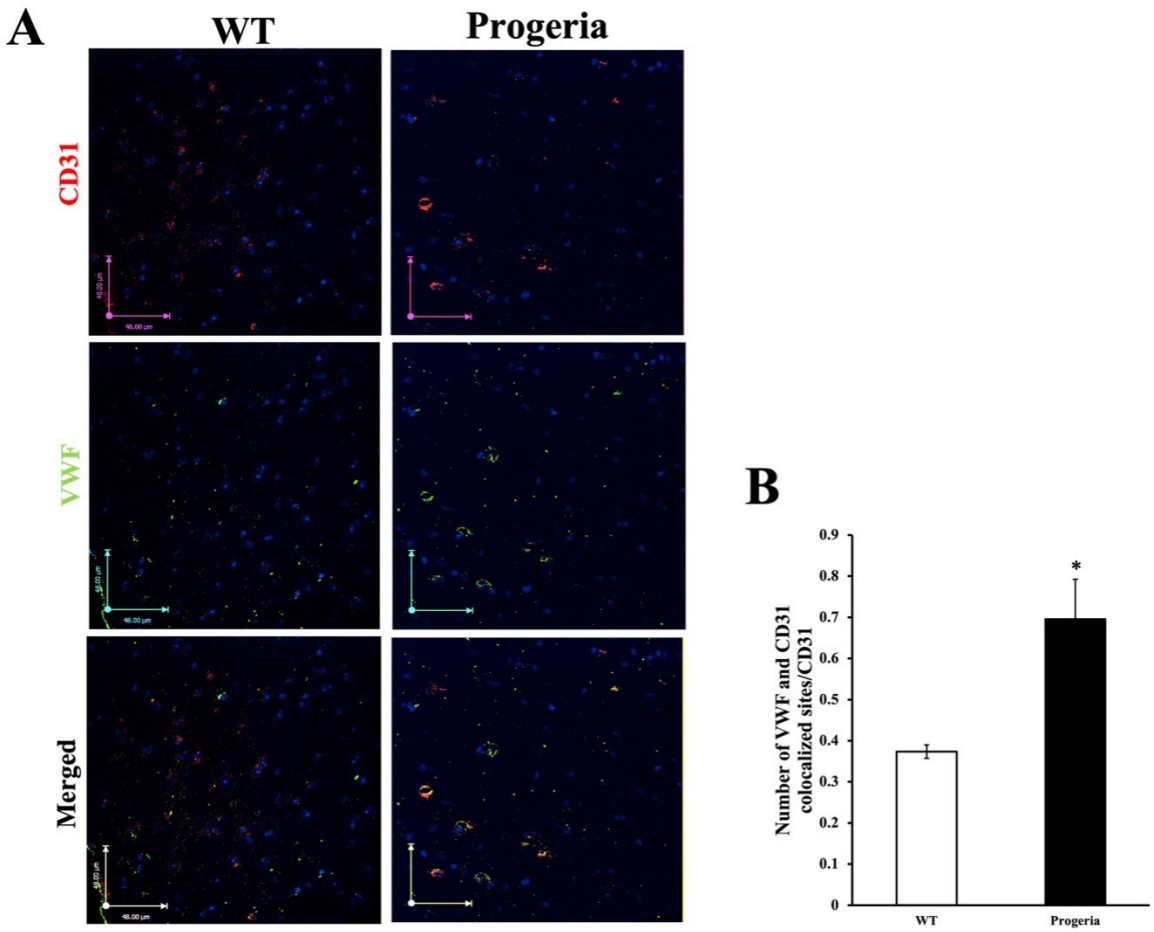


Figure 4.1.3. Immunofluorescent and confocal microscopy analyses of VWF and CD31 in the brain section of WT and progeria mice.

(A) Immunofluorescence co-staining for VWF (green) and CD31 (red) were performed on the brain sections of WT and progeria mice. Colocalization of VWF with endothelial cell marker

(CD31) is detected as yellow in merged images. Cell nuclei were visualized using DAPI (blue). Scale bar is 48 μm. (B) Quantitative analysis of the number of micro vessels that express VWF and CD31 as a fraction of total CD31 expressing microvessels per field of view. Three field of view were analyzed per animal (n=3).

4.1.4 Cell Senescence and VWF expression in progeria mice

To investigate whether cell senescence contributes to VWF expression in progeria mice, immunofluorescence co-staining with specific antibodies against senescent marker (SA-β-Gal) and VWF was performed in the brain sections of both progeria and WT littermates. The co-localization of VWF and SA-β-Gal was significantly higher in endothelial cells of progeria than in WT mice. Only a small proportion of VWF-expressing endothelial cells exhibited senescence in WT mice (Figure 4.1.4 A-B)

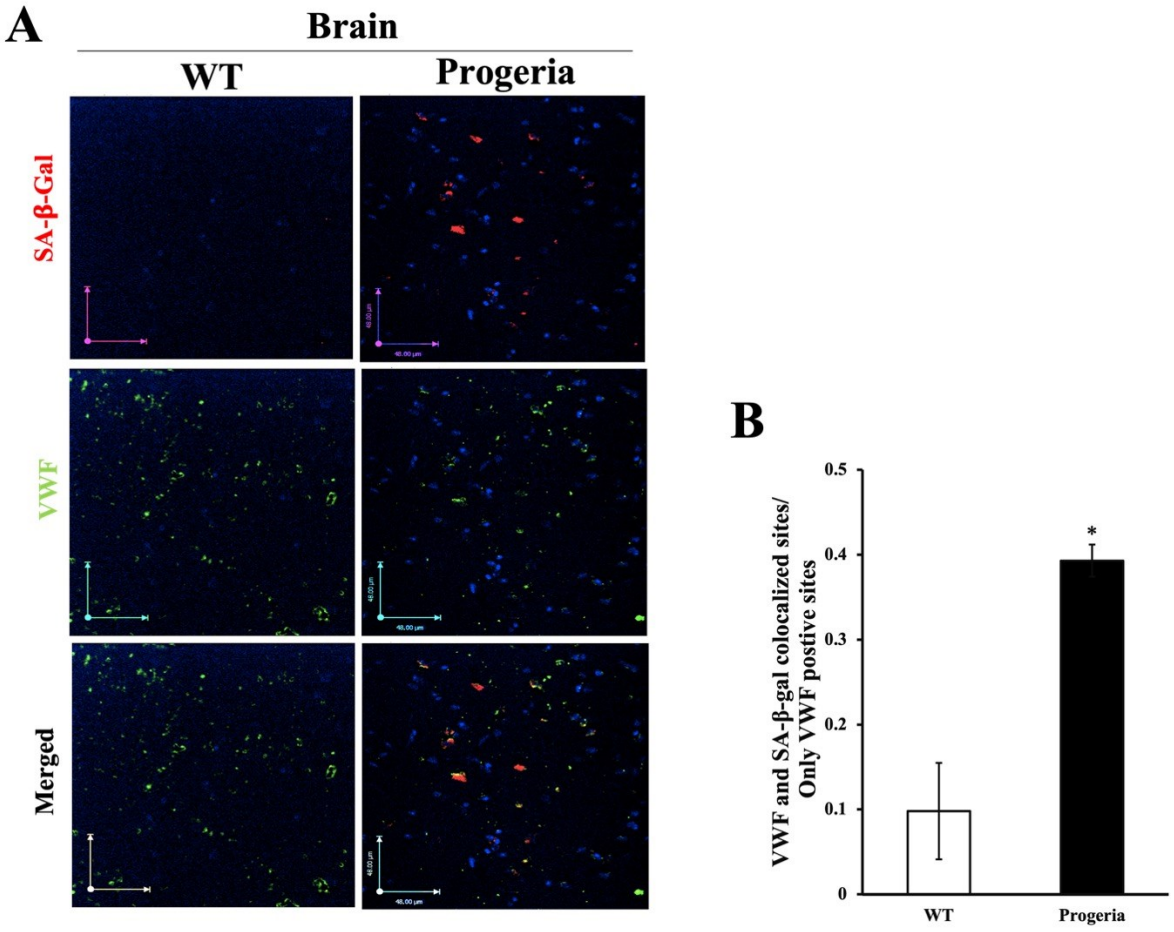


Figure 4.1.4. Immunofluorescent and confocal microscopy analyses of VWF and SA-β-Gal in the brain section of WT and progeria mice.

(A) Confocal microscopy images of co-immunofluorescence staining of the brain sections of WT and progeria mice to detect VWF (green) and SA-β-Gal (red). (B) Quantitation of the average number of colocalization sites of VWF and SA-β-Gal as a ratio of total VWF expressing microvessels. Three field of view were analyzed per animal (n=3).

4.1.5 Targeted reduction of senescent endothelial cells in the brain using senolytic drug

To explore whether targeted reduction of senescent endothelial cells *in vivo* can abrogate VWF upregulation and consequently reduce platelet aggregate formation in aged mice, in collaboration with Dr. John Lewis's group, the aged mice were treated with a senolytic drug and

platelets aggregate formation and senescent endothelial cell proportions were determined in the brains. This drug consists of a lipid nanoparticle (LNP) that encapsulates a DNA plasmid encoding caspase 9 under the control of the p53 promoter, which activates the apoptosis cascade in the target cell population. In other words, the drug specifically targets senescent cells expressing p53 for destruction. Aged mice (18 months old) were treated with 200 μg of the senolytic drug, or PBS as control, for two weeks/ once per day. Brains were collected from these mice as well as young mice (2.5 months) for IF analyses to detect platelet aggregates, as well as SA-β-gal and CD31 colocalization sites.

In addition to vascular endothelial cells, the brain contains various non-endothelial cells, including neurons, glial cells, and astrocytes. SA-β-Gal staining is not specific to endothelial cells; it is a general marker for any senescent cell. To assess the efficacy of the p53-Casp9 drug in specifically targeting senescent endothelial cells among all other senescent cells in the brain, we performed immunofluorescence staining against CD31 (an endothelial cell marker) and SA-β-Gal (Figure 4.1.5 A-B). The results demonstrated a higher percentage of senescent endothelial cells in control aged mice (treated with PBS) compared to young. However, after treating aged mice with the senolytic drug (p53-Casp9-LNP) there was a significant reduction in the percentage of senescent endothelial cells. These data demonstrated that the drug treatment effectively targeted and significantly reduced senescent endothelial cells.

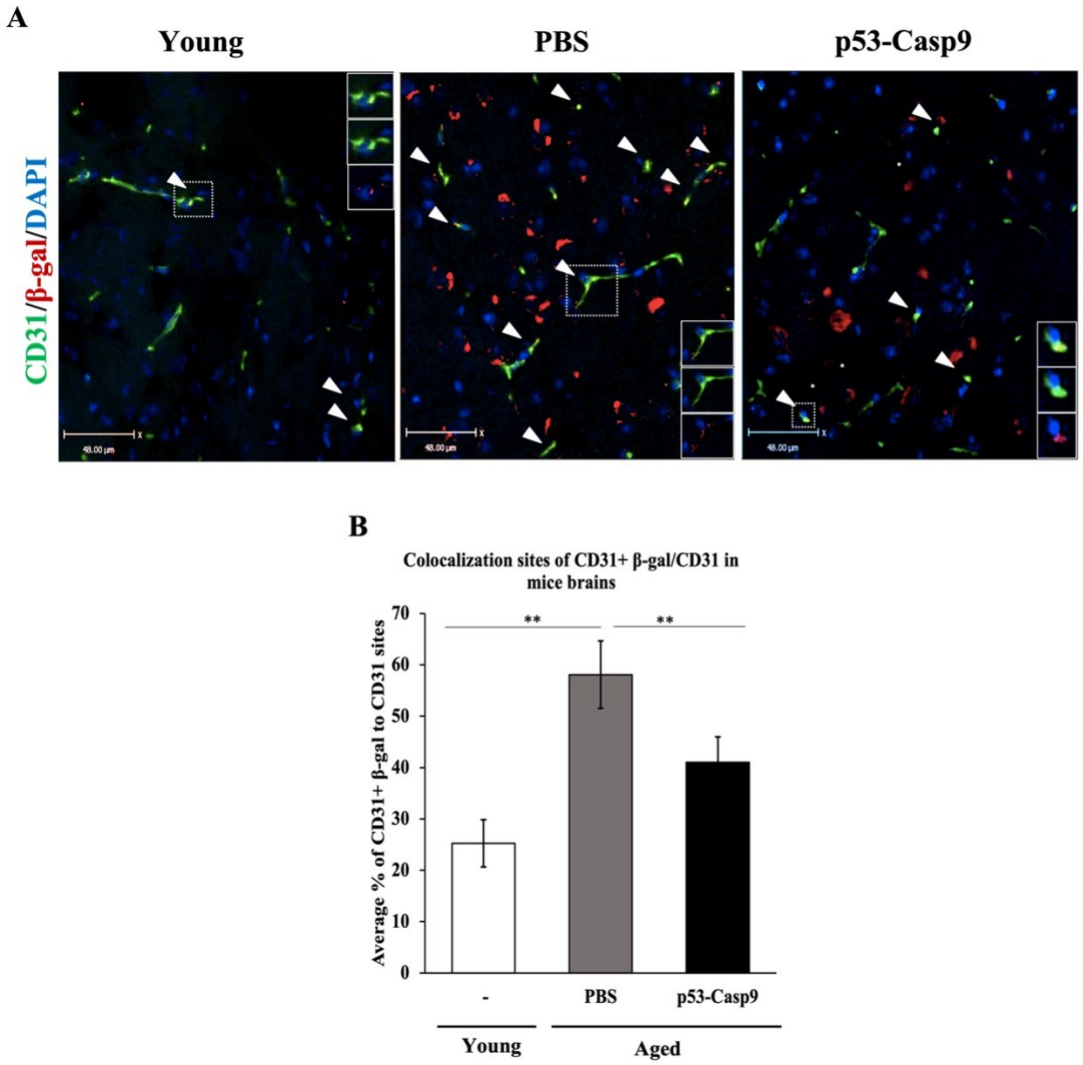


Figure 4.1.5. Quantification of senescent endothelial cells using CD31 and SA-β-gal staining.

(A) Immunofluorescence analysis to identify CD31 (green) and SA-β-gal (red) expressing cells. Representative cells with co-localized CD31 and SA-β-gal expression are indicated by white arrows. Insets indicate individual channels for the selected region within the white rectangle, highlighting colocalization. Scale bar is 48 µm. (B) Graph showing the quantitative analysis to determine the percentage of endothelial cells displaying senescence marker by calculating the ratio of CD31+ SA- β -gal/CD31.

Next, to determine the functional consequence of using the senolytic drug, the brain sections of treated and control aged mice were assessed for platelet aggregates by IF staining with anti CD41 as previously described above (Chapter 3 Figure 3.1.4). The results demonstrated that aged mice treated with LPN (p53-Casp9) exhibited a significant reduction in platelets aggregate formation in the brain vasculatures compared to control sham-treated aged mice (PBS). **(**Figure 4.1.6 A-B). This result suggests that senescent endothelial cells contribute to enhanced platelet aggregate formation and is consistent with the hypothesis that senescent-induced VWF upregulation has functional consequences regarding enhanced platelet aggregate formation. Developing anti-senolytic or small molecules, which can target senescent cells specifically through p53, could be a potential therapeutic approach for combating age-related vascular/microvascular dysfunction.

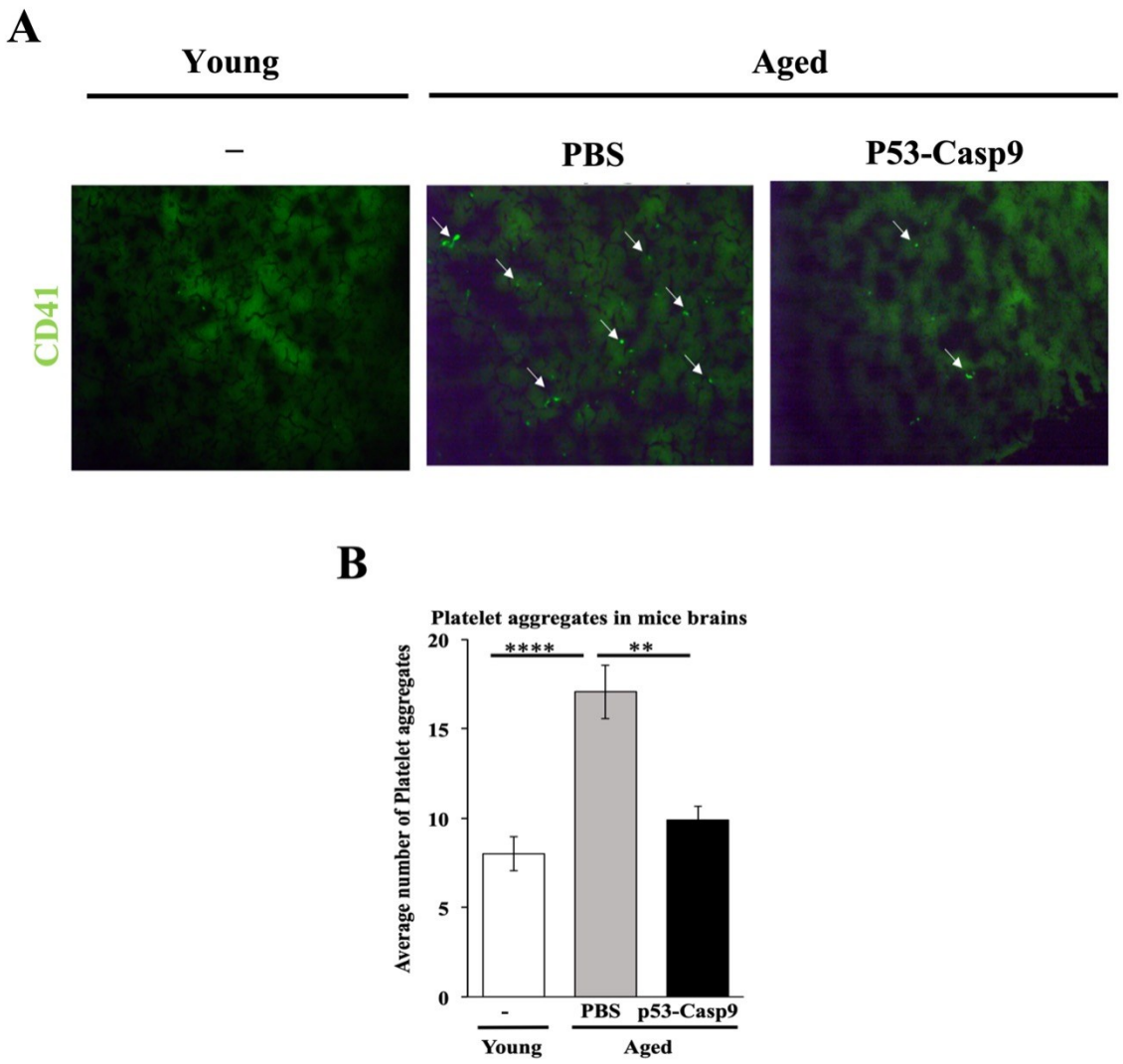


Figure 4.1.6. IF staining to detect platelet aggregates in treated aged mice compared to control and young (untreated) mice.

(A) Aged mice (18 months old) were treated with p53-Casp9 LNP or PBS and IF staining, using CD41 antibody (green), is performed to detect platelets aggregates (representative shown by arrows) and (B) quantification analysis is shown in bar graph.

5 Chapter 5

5.1 Lung physiology

The lung serves as the primary organ of the respiratory system. The right lung is divided into three distinct lobes, while the left lung is divided into two lobes. The left lung is marginally smaller than the right lung due to the spatial accommodation of the heart on the left side. Upon inhalation, air navigates through the airways and descends into the alveoli, or air sacs, within the lungs. It is within these alveoli that the critical process of gas exchange occurs [359].

The blood vessels of the pulmonary circulation are the pulmonary arteries, veins and microvessels [360]. The pulmonary arteries transport deoxygenated blood from the right side of the heart to the lungs for oxygenation. This exposes lung tissues consistently to a significant level of hypooxygeneation compared to other organs. Based on our report that hypoxia specifically upregulates VWF expression, we hypothesize that this hypooxygenated environment of pulmonary arteries may partly contribute to high basal level of VWF mRNA in lung compared to other organs. While highly vascularized nature of lung is also a major contributing factor to relatively high VWF mRNA levels. The right and left pulmonary arteries specifically provide blood flow to their respective lungs and further divide into the lobar, segmental, and sub-segmental arteries within the lungs, eventually forming capillaries around the alveoli. Since pulmonary circulation is characterized as a system with high capacitance and low pressure, it is ideal for effective gas exchange [361]. During respiration, carbon dioxide is released from pulmonary arteries, and oxygen is picked up. Pulmonary veins carry the oxygenated blood from the lung and return it to the left part of the heart to complete the pulmonary cycle [361].

5.2 General introduction into lung transplantation

In the 1940s and 1950s, animal experimentation by Demikhov and Metras demonstrated the feasibility of lung transplantation procedures [362]. In 1963, Hardy performed the first human lung transplantation. The donor was deceased due to cardiac death, and the left lung transplant was performed on a recipient who survived only 18 days [363]. From 1963 to 1978, large varieties of attempts at lung transplantation were unsuccessful because of rejection and anastomotic bronchial and tracheal healing complications. In the 1980s, the introduction of a powerful immunosuppressant, cyclosporin A, generated renewed interest in organ transplantation, including lung transplantation [364]. It was thought that lung transplantation would never become a possible therapeutic choice because of so many failures. However, in 1981, Bruce Reitz and Norman Shumway performed the first successful combined heart-lung transplant at Stanford in a 45-year-old woman with end-stage primary pulmonary hypertension [365]. This operation set the stage for the possibility of future isolated lung transplantation in humans. 1986, Dr. Cooper at the University of Toronto performed the first successful single lung transplant [366]. After that, in 1988, Dr. Alexander Patterson designed the technique of double-lung transplantation [367]. Advances in surgical techniques, lung preservation, immunosuppression, and management of infections have influenced the improvements in mortality and reduced post-transplant complications. Lung transplantation is a complex process, and the threat of complications is high. Some complications are associated with the surgery itself, while others may result from the immunosuppressive treatment, underlying diseases, organ preservation method or other unidentified causes which is necessary to stop the rejection of the donor lungs.

5.2.1 Complication after lung transplant: Thrombogenicity

Lung transplantation has become an effective treatment for patients with varieties of end stage lung diseases such as emphysema, fibrosing alveolitis, cystic fibrosis, pulmonary hypertension and bronchiectasis [368-371]. Patients who survive the transplantation wait time have to endure the menace of potential complications before receiving the benefits of transplanted organs [372].

The most prominent complications of lung transplantations is microthrombus formation [114]. Organ transplantation has developed and reached its todays' state as an effective treatment as a result of scientific and clinical advances in surgery, patient selection, management, and availability of potent immunosuppressive agents. One of the most important factors in the improvement of transplantation is organ preservation protocol.

5.2.2 Lung preservation before transplantation

Implementing organ preservation protocols is crucial in enhancing transplantation outcomes. These protocols ensure the delivery of high-quality donor organs via a suitable organ exchange system, which aligns the most suitable recipient with the best available organ. This advancement has been made possible by understanding the impact of hypoxic injury on donated organs and developing specific organ preservation techniques to mitigate its harmful effects.

5.2.3 Approaches to organ preservation

5.2.3.1 Static cold storage (SCS)

With the limitation of organs available for transplant, there is a great need for better use of available organs. An essential step in the transplant process is organ preservation. The main criterion in organ preservation is to conserve the proper function of the organ and tissue during

storage so that the transplanted organ will function optimally after reperfusion. The two current preservation methods for most organs before transplantation are static and dynamic.

The principal method for static storage is simple static cold storage (SCS); in this technique, the lung is cooled and perfused with a preservation solution, then inflated with oxygen and stored on ice [373]. This method is well-established and universally accepted for preserving organs [374]. It is relatively simple and cost-effective [375]. However, the preservation time with this method is generally limited to around 6 hours [376]. It may be less effective in maintaining the functional integrity of the organ over an extended period. SCS preservation of organs may lead to ischemic injury and other complications, including those arising from extended hypoxia.

5.2.3.2 Ex vivo lung perfusion (EVLP)

The other perfusion-based methods, such as normothermic machines, provide techniques for dynamic preservation [374]. This emerging technology has great potential to increase the donor lung supply. In this method, the lungs are kept warm and breathing, similar to conditions in the human body. This technique can extend the preservation time beyond what is possible with SCS. It can help to improve the quality of the donor organs before transplantation. In addition, this method has been shown to prevent ongoing injury associated with prolonged ischemia and accelerated lung recovery [377]. Despite its advantages, there are some difficulties with this preservation method, including the requirement of specialized equipment and trained personnel. It is generally more expensive than SCS, and it is still in the early stage of clinical adaption and has yet to be universally accepted for all organs [378].

5.2.3.3 EVLP device description

Various transplant groups have started using EVLP in the clinical setting using their assembled homemade system. The circuit includes different parts such as a centrifugal pump, heater/cooler, tubing, hard-shell reservoir, hollow-fibre oxygenator, leukocyte filter, in-line gas analyzer, saturation probes, and pressure transducers [379]. The Heater/Cooler Unit (HCU) delivers water at a fixed temperature that flows into the heat exchange device during EVLP to create the normothermic environment. During ex vivo perfusion, an intensive care unit (ICU) ventilator controls the pressure that ventilates the lungs. It allows the user to pre-set pressure and volume limits, thus preventing ventilator-induced lung injury [379].

To provide a suitable condition (warm and humid) and to maintain the organs during ventilation, the lungs are placed in a special plastic chamber (Figure 5.2.1). The perfusate leaves the lungs through the left atrium (LA) cannula and enters the reservoir. From there, using a centrifugal pump the perfusate is pumped into the oxygenator and heat exchanger. The perfusate is deoxygenated through a special gas tank consisting of a low oxygen gas mixture (86% N2, 8% CO2 and 6% O2) and warmed to normothermia in the heat exchanger. The perfusate then flows through a leukocyte filter before recirculating in the lungs via the PA cannula for oxygenation. The Catheters and pressure transducers measure pulmonary artery pressures (PAP) and left atrial pressures (LAP). During the perfusion, a temperature probe is used to screen the circuit temperature, and flow probes measure PA and LA perfusate flow. Lastly, lung ventilation starts with a standard care unit ventilator (Figure 5.2.1).

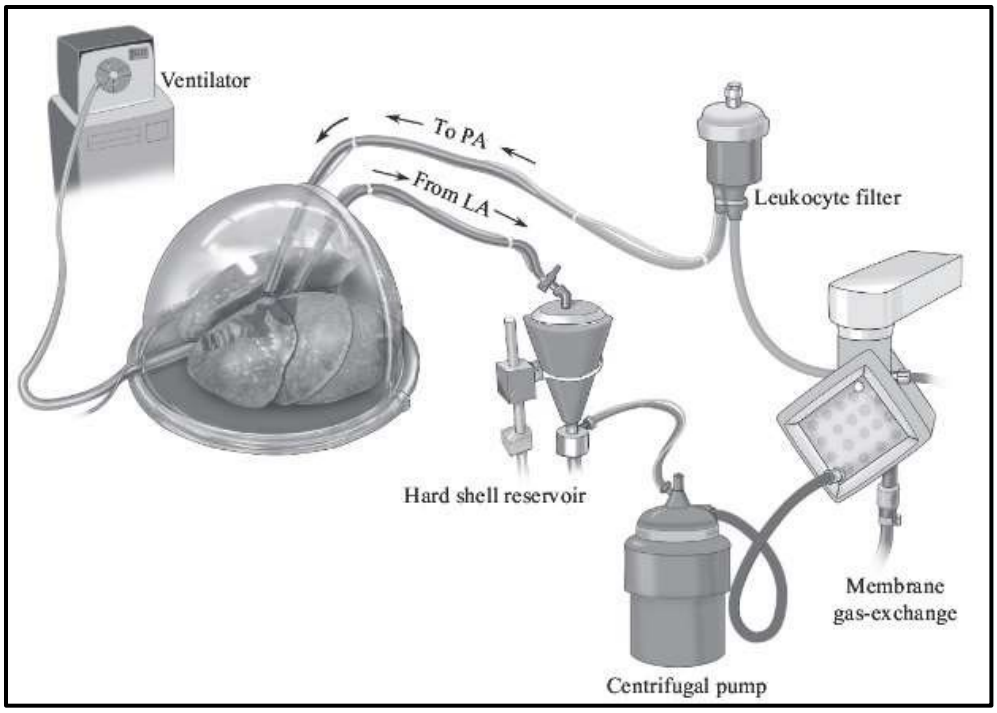


Figure 5.2.1. Schematic of the standard ex vivo lung perfusion circuit

The image reproduced from Cypel et al. [380]

The manual setup of the EVLP circuit is time-consuming and requires experienced personnel to monitor the graft for several hours. The whole transplant procedure, including donor assessment, lung retrieval, EVLP, lung transplantation, and safe recipient transport to the ICU, can keep various team members busy for nearly a full day. Therefore, further standardization of the technology and techniques is needed to simplify EVLP practice. Ventilation type in the EVLP setting can be either positive pressure ventilation (PPV) or negative pressure ventilation (NPV) [381-384].

5.2.3.4 Different ventilation methods in EVLP setting

Positive pressure ventilation (PPV) is like using a mask or ventilator to deliver air into the lungs [385]. Similarly, PPV pushes air into the lungs by creating positive pressure. PPV is a

well-established method for ventilating lungs during EVLP, although it may lead to ventilator lung injury, which is a concern during EVLP [386].

On the other hand, there is a negative pressure ventilation (NPV) technique. NPV method is similar to the physiological condition [387]. When we breathe in, the chest expands and creates a negative pressure (vacuum), which pulls air into the lungs. NPV mimics our natural breathing feature during artificial ventilation. NPV may reduce ventilator lung injury compared to PPV[382, 383, 388]. It is a newer technique and may need to be better established as PPV. While it may be more effective than PPV in preserving lung function and reducing injury during EVLP, more research and investigation are required to fully understand its long-term effects and potential benefits.

5.2.3.5 Cellular/Acellular Perfusate in EVLP setting

The widely utilized acellular EVLP perfusate is Steen solution, which contains buffered electrolytes, glucose, Dextran-40, and albumin [389]. Acellular Steen perfusate has been observed to suppress the activation of pro-inflammatory signalling in human lung endothelial and epithelial cells [390].

On the other hand, cellular perfusate can largely prevent ischemia *ex vivo* reperfusion-associated lung injury, and there is less intra-alveolar oedema (fluid accumulation) detected in cellular EVLP than in acellular EVLP [391]. There are several settings for EVLP regarding ventilation, perfusate types and components, but ongoing research and clinical validation are essential to confirm its effectiveness [392]. During organ transplantation, specifically CSC, the donor organ is exposed to hypoxia for a while, and based on our observation that hypoxia results in increased levels and altered expression pattern of VWF, we hypothesized that hypoxia/ischemic

conditions of the donor's lungs during the transplantation procedure may result in increased VWF expression. This may consequently contribute to increased thrombogenicity and potentially thrombus formation and, consequently, organ dysfunction. The reduced hypoxia exposure under EVLP condition may ameliorate/ reduce VWF upregulation and consequently decrease thrombotic complication.

5.3 Determining the role of von Willebrand factor in thrombotic post-transplantation complications using ex vivo lung perfusion system

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5.3.1 Abstract

Von Willebrand factor (VWF) is a pro-coagulant glycoprotein expressed only in endothelial cells and megakaryocytes. It mediates adhesion of platelets to the endothelial/sub-endothelial surfaces, which is the primary step in thrombogenesis. External stimuli, including hypoxia was shown to upregulate VWF expression levels, and alter its vascular tree expression pattern in lung. Increased VWF levels, is a significant risk factor for thrombus formation, which is a major complication of organ-transplantation. Since during organ-transplantation donor organs are under hypoxic conditions, we explored whether this may lead to alterations in VWF expression, and whether modification of transplantation procedure to reduce hypoxic exposure could prevent such alterations. Procured pig's lungs that were maintained in static cold storage "SCS" or exposed to warm perfusion were used. Lung tissue biopsies were obtained immediately after organ harvest, 12 hours post cold storage, or post warm perfusion. VWF RNA and protein expression levels and patterns were analysed using RT-PCR, western blot and immunofluorescent analysis. Immunofluorescent analysis demonstrated that transplanted lungs that were maintained under SCS, but not EVLP, exhibited VWF expression in an increasing number of microvascular endothelial cells while, warm perfusion led to a significant reduction in the levels of VWF mRNA and protein expression compared to cold storage.

5.3.2 Introduction

Organ transplantation, including lung transplant, has become an effective therapeutic option for patients with varieties of end-stage diseases [393-395]. However, its application is limited because of the shortage of suitable donor organs [396, 397]. Disparity between the supply of transplantable lungs and demand of potential recipients leads to long waiting times and annual rises in deaths of patients on the lung transplant waiting lists. Consequently, attempts have been focused on use of living-donors, marginal donors, and non–heart-beating donors to expand the donor pool [398]. However, in addition to the problem of supply and demand, patients have to overcome the threat of potential complications from transplantation procedures, including microthrombus formation, which may lead to early allograft failure.

Microthrombus formation is one of the major complications in lung transplantation [114]. The coagulation process that contributes to vascular thrombosis depends on alterations in the ratio of pro to anticoagulant factors, in favor of the pro-coagulant molecules [10, 399]. Hypercoagulable state is susceptible to thrombus formation; and a pro-coagulant molecule, namely von Willebrand factor (VWF), has a major role in this process. VWF is a multimeric, pro-coagulant glycoprotein that is the primary initiator of thrombus formation. It is produced only in endothelial cells (ECs) and megakaryocytes [138, 305]. In the site of injury VWF molecules attach to the exposed subendothelial layer and capture platelets to form platelet aggregates, which seals the damaged vessels' wall [135, 320, 400]. In addition, even in the absence of injury, but in response to some stimuli, VWF can be released from ECs and attach to the luminal cell surfaces of the intact endothelium [401]. Under these circumstances also, VWF can capture platelets and form platelet aggregates, which may be subsequently stabilized through fibrin deposition, leading to thrombus formation [11, 33, 402]. It has been reported that hypoxic condition, as a stimulus can upregulate

VWF expression [41]. Thus, this hypoxia driven VWF upregulation may contribute to increased thrombogenicity [41]. Increased levels of VWF have been reported in patients with pulmonary hypertension (PH), which results from shortage of oxygen in lungs [403, 404]. Organ transplantation is among conditions that lead to hypoxia exposure of organs as a result of extended disconnection of the donor organs from circulation [405]. Thus, considering that thrombosis is a major complication of organ transplantation, we hypothesized that hypoxia-induced VWF upregulation may contribute to thrombotic complications during organ transplantation.

Previously we have reported that in mice, hypoxia exposure upregulates VWF expression in all major organs including brain, heart, liver and lung but not kidney [41]. Furthermore, specifically in lung, (but not vascular beds of other organs) hypoxia resulted in de novo activation of VWF expression in microvascular endothelial cells, where normally VWF expression is not detected. We also demonstrated increased platelet adhesion and aggregates formation, as a functional consequence of hypoxia-induced VWF upregulation [41].

One of the most important factors in the improvement of transplantation is an optimal organ preservation protocol to conserve proper function of the donor organ during storage so that the transplanted organ will function optimally after reperfusion [373, 406]. Current standard method for organ preservation, is static cold storage (SCS) [374, 407]. This method involves cold ischemia, which leads to extended hypoxic condition due to prevention of oxygen supply to the donor organ for a period of time prior to transplantation [405]. However, *ex vivo* lung perfusion (EVLP) is a novel perfusion-based technique for dynamic lung preservation, which significantly reduces the hypoxia exposure of the donor lung [381, 391, 408]. In this method, risk of exposure to the cold ischemia and hypoxic condition is minimized by circulating oxygenated, nutrient-rich perfusate to

the lung during preservation. We hypothesized that the method of organ preservation may influence the level of hypoxia exposure of the donor lungs during transplantation. This may in turn impact VWF expression, and consequently the propensity for thrombogenic complications. In this study we propose to determine whether VWF expression at the mRNA and protein levels, as well as vascular tree distribution pattern of VWF, are altered in lungs, which were kept in SCS condition, and whether the procedure of warm perfusion moderates this effect.

5.3.3 Material and methods

All animal experiments in this study were performed under an approved protocol by the University of Alberta Institutional Animal Care Committee. All animal care and use were in accordance with the guidelines of the Canadian Council on Animal Care, and the Guide for the Care and Use of Laboratory Animals.

5.3.3.1 EVLP experimental groups

EVLP were performed using two types of ventilation mode [Positive Pressure Ventilation (PPV) or Negative Pressure Ventilation (NPV)] and the two types of perfusate composition materials were used [Acellular or packed red blood cells (pRBC)]. Twelve porcine lungs from female domestic pigs (42 ± 5 kg) were collected and allocated into four different experimental groups with three animals per group (PPV +Acellular, PPV+pRBC, NPV+Acellular, NPV+pRBC).

5.3.3.2 Ex vivo lung perfusion condition

Lungs were perfused under normothermic ex-vivo perfusion (EVLP) for 12 hours as described previously [381]. Briefly, for making the perfusate solution, freshly standard Krebs-Henseleit buffer (KHB) solution was made for experiments. 1.5L of (KHB) with 8% bovine plus 0.5L of red blood cell concentrate were used as a cellular perfusate (RBC). The acellular perfusate

solution was created of 2L KHB with 8% BSA. Under PPV-EVLP platform, donor lungs were ventilated by support of a standard intensive care unit (ICU) ventilator (SERVO-I, Maquet Critical Care AB, Solna, Sweden). While under NPV-EVLP platform, to make a negative pressure inside of sealed organ chamber, a custom-built turbine driven ventilator was used to produce the normal tidal volume.

5.3.3.3 Sample collection

Tissue biopsies were obtained immediately prior to static cold storage or normothermic *ex vivo* perfusion as (T0) samples. Lung tissue biopsies were obtained after 12 hours post cold storage, or post warm perfusion as (T12) samples. Samples were immediately snap frozen by immersing in liquid nitrogen and kept at -80 C for further analysis. VWF RNA and protein expression levels were analyzed using RT-PCR and western blot.

5.3.3.4 VWF protein levels assessment

Tissue biopsies were obtained from lungs in vivo, after harvest, and EVLP or SCS treatment. All samples were collected from peripheral regions of lungs. Samples were immediately snap frozen by immersing in liquid nitrogen and kept at -80 C and subjected to Western blot analyses as follows.

Protein extraction and determination of protein concentration, and western blot analyses were performed as previously described [41]. Briefly, Protein samples (25 μg) were separated on 6% sodium dodecyl sulfate polyacrylamide gel and were transferred to a polyvinylidene difluoride membrane. Rabbit anti-VWF, and anti-tubulin were used as primary antibodies. HRP-conjugated goat anti-rabbit antibody was used as the secondary antibody. Tubulin was used as the loading control. Bands were detected and analyzed on a Bio-imaging System as previously described.

5.3.3.5 Quantitative reverse transcriptase-polymerase chain reaction

Total RNA was isolated from frozen lungs tissues using the RNeasy Mini kit. cDNA was synthesized using the High Capacity RNA-to-cDNA Master Mix. RT-qPCR was performed using SYBR Green master mixes (Life Technologies) with ABI7500 Real-Time PCR system. All PCR reactions were repeated in triplicate. Bio-Rad PrimePCR SYBR Green Assay VWF and HPRT primers were used for RT-PCR.

5.3.3.6 Immunofluorescence

For immunofluorescent analyses lung sections were snap frozen in OCT and sectioned in 5 μm sections. Immunofluorescent analyses were performed using primary sheep anti-VWF and rabbit anti-CD31 antibodies. Alexa Fluor 568 donkey anti-sheep IgG (H+L) and Alexa Fluor 488 donkey anti-rabbit IgG (H+L) were used as a secondary antibodies respectively. The tissues were incubated with the primary antibody overnight at 4℃, washed with PBS and then incubated with the secondary antibody for 1 h at room temperature. Images of the slides were captured using a WaveFX confocal microscope from Quorum Technologies microscope.

5.3.3.7 Statistical analysis

GraphPad Prism 6.0 was used for the statistical analysis. Data are expressed as the mean ± SEM. The paired, two-tailed Student's t-test was used for the comparison between means. $P<0.05$ was considered to indicate a statistically significant difference.

5.3.4 Results

5.3.4.1 Alteration of VWF mRNA levels and protein in lungs that are maintained under cold storage

To determine whether standard procedures of cold preservation of organs influences the level of VWF expression, we used pig's lungs, which were harvested and maintained under static cold storage (SCS) for 0 or 12 hours. RNAs were prepared from 6 independent organs at 0 (T0) and 12 (T12) hours SCS and subjected to quantitative RT-PCR analyses to detect and compare VWF mRNA levels. The results demonstrated that VWF mRNA levels (normalized to HPRT) were either significantly increased or remained unchanged after 12 hours of SCS compared to 0 hours (Figure 5.3.1)**.** The average fold induction, although not statistically significant, among 6 samples were 1.5 (Figure 5.3.1). Results for individual samples presented in supplementary data (Figure 5.3.9).

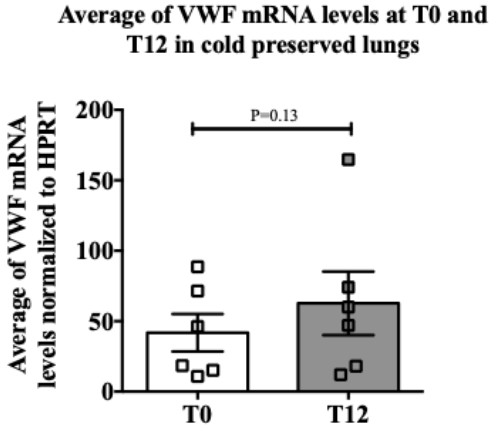

Figure 5.3.1. Analysis of VWF mRNA levels at T0 and T12 from SCS preserved lungs.

Quantitative real time PCR analysis was used to determine the mRNA levels of VWF, normalized to HPRT mRNA, in lungs samples at T0 (white bar) and after 12 hours in SCS (T12) (black bar).

Average levels of VWF mRNA were reported for the 6 independent experiments (n=6 per group). Data are shown as the mean ± SEM. (*$p < 0.05$).

Next, to determine whether unaltered or increased VWF mRNA levels are translationally reflected in protein levels, we performed Western blot analyses on 5 pairs of the samples, which were used for RNA analyses. The results demonstrated that there was a trend towards increase in cellular VWF protein levels (normalized to tubulin) in samples maintained under SCS condition for 12 hours (T12) compared to 0 hours (T0), and the average ratio of increase was 1.2 (Figure 5.3.2 A-B). However, the levels of increases were not statistically significant (Figure 5.3.2 B)**.**Results for individual samples presented in supplementary data (Figure 5.3.10).

Cold-induced suppression of activities of cellular machinery, including transcription and translation is expected to generally result in a significant reduction in levels of various cellular mRNAs and proteins. However, these results demonstrated that this does not pertain to VWF. VWF mRNA and protein levels were either maintained or demonstrated an increasing trend in levels in response to extended time period under cold storage.

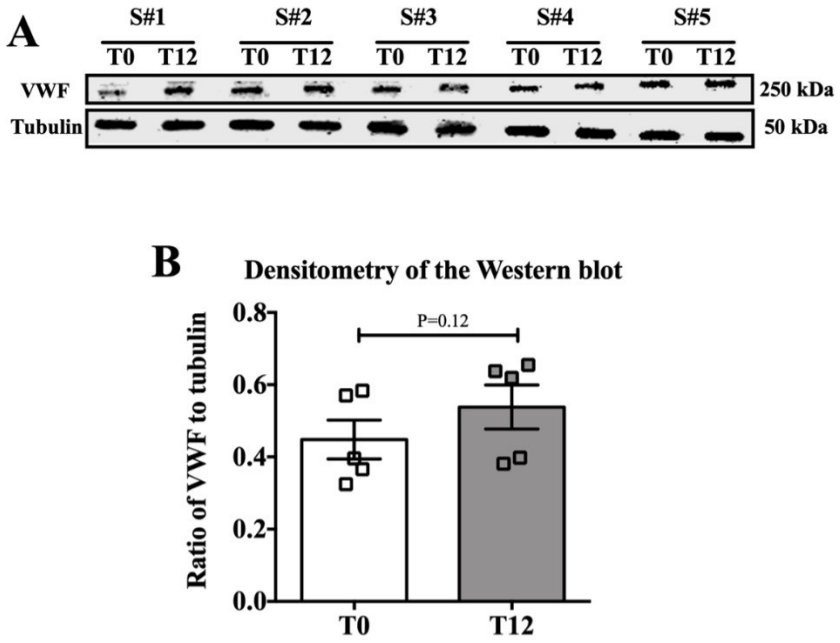

Figure 5.3.2. Analysis of VWF protein levels at T0 and T12 from SCS preserved lungs.

(A) Western blot analyses were performed to detect VWF protein in preserved lungs at T0 and in SCS condition for 12 hours (T12). Tubulin was used as normalization control. (B) The graphs represent densitometry quantification after normalization to tubulin averaged from 5 independent animals (n=5 per group). Data are shown as the mean ± SEM. (*$p < 0.05$)

5.3.4.2 VWF expression pattern at SCS preserved lungs prior and post transplantation

Our previous analyses of hypoxia-induced alterations in VWF expression in mice lungs demonstrated that there was a significant increase in proportion of lung microvascular endothelial cells that exhibited de novo activation of VWF expression (19). Thus, we proceeded to determine whether SCS preservation also alters the proportion of pig's lung vascular endothelial cells that exhibit VWF expression. For these analyses, we performed double stained immunofluorescence analyses using CD31 (endothelial cell marker) and VWF antibodies on samples of lungs that were preserved and transplanted. These included IV (in vivo pre transplant), PT0 (immediately at the

beginning of transplant and after 12 hours at SCS condition) and PT4 (4 hours post-transplant and after 12 hours maintenance in SCS) lungs.

The data demonstrated that there were significantly increasing number of small vessels (indicated by CD31 staining) that exhibited VWF expression at PT4 compared to IV and PT0 (Figure 5.3.3 A)**.** Quantitative analysis was performed and reported as a ratio of VWF and CD31 positive sites (indicative of vessels that express VWF) to the total CD31 positive sites (representing total number of vessels) per field of view (Figure 5.3.3 B)**.** These results demonstrated that preservation of lungs under cold storage conditions prior to transplantation alters VWF expression pattern leading to increasing number of microvascular endothelial cells that express VWF.

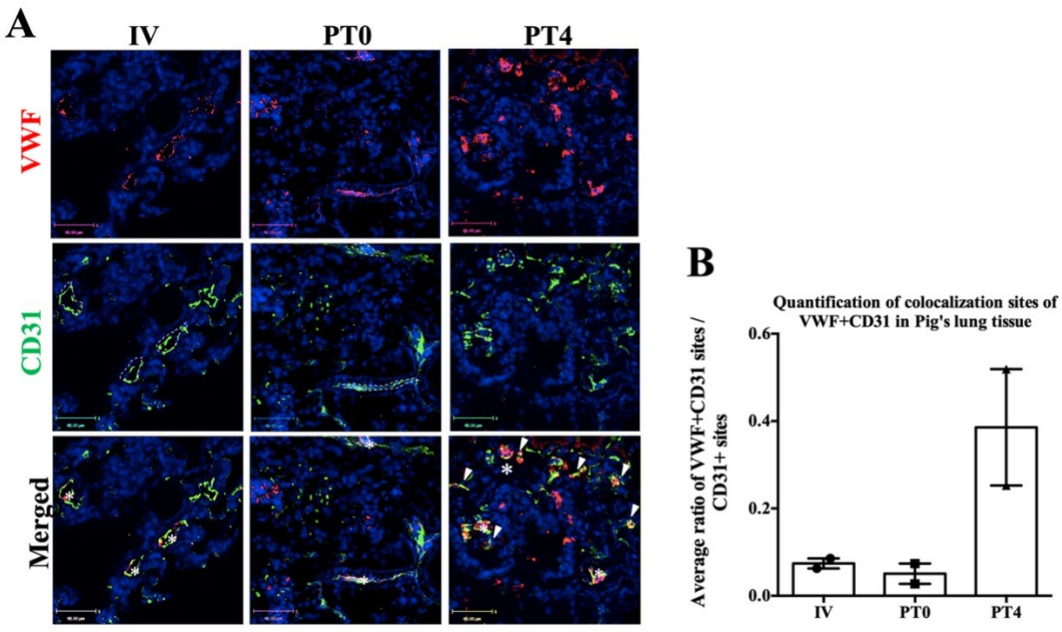

Figure 5.3.3. Immunofluorescent analysis of lungs that were kept in SCS before and after transplant.

(A) Immunofluorescence analysis were performed using double staining for CD31 (green) and VWF (red) on pig's lung samples as described in Material and methods. (IV: in vivo pretransplant,

PT0: immediately at the beginning of transplant and after 12 hours at SCS, PT4: four hours post transplantation and after 12 hours storage at SCS). * Represents colocalization of VWF and CD31 in a large/medium vessel. Arrows indicate colocalization of VWF and CD31 in small vessels. Representative large/medium vessels are marked with dotted lines in the image. Scale bar is 48 μm. (B) Quantification analysis was performed to identify the ratio of VWF expressing CD31 positive vessels to total CD31 positive vessels based on analyses of 4-5 fields of view of lung sections from 2 pigs for each group (n=2). Data are shown as the mean ± SEM. (*$p < 0.05$)

5.3.4.3 Alteration of VWF mRNA levels and protein in lungs that are maintained under warm perfusion

It has been reported that using positive pressure ventilation (PPV) in normothermic ex-vivo lung perfusion (EVLP) setting, with both acellular and red blood cell (RBC) based perfusate solutions, have improved the rate of transplantable organs procurement [381, 382]. In addition, previously our group showed utilization of negative pressure ventilation (NPV) during EVLP, which is similar to physiological condition can improve the outcome, regardless of perfusate solution composition [382]. To see if exposure to warm perfusion (instead of SCS) has any effect on the levels of VWF expression, the samples that were exposed to warm perfusion [with either acellular or RBC perfusate solution and maintained on normothermic condition (either PPV or NPV)] were tested for VWF mRNA and protein expression levels.

Tissue samples were collected from harvested lungs immediately prior to perfusion, and after 12 hours of perfusion, and used for RNA preparation and RT-PCR analyses to detect VWF mRNA as described above for SCS lung samples. The results demonstrated that VWF mRNA levels were significantly reduced in most of the EVLP conditions at T12 compared to T0 (Figure

5.3.4**).** Under EVLP condition that included NPV with acellular perfusate, although the average level of alteration was not statistically significant, there was a clear trend towards reduction.

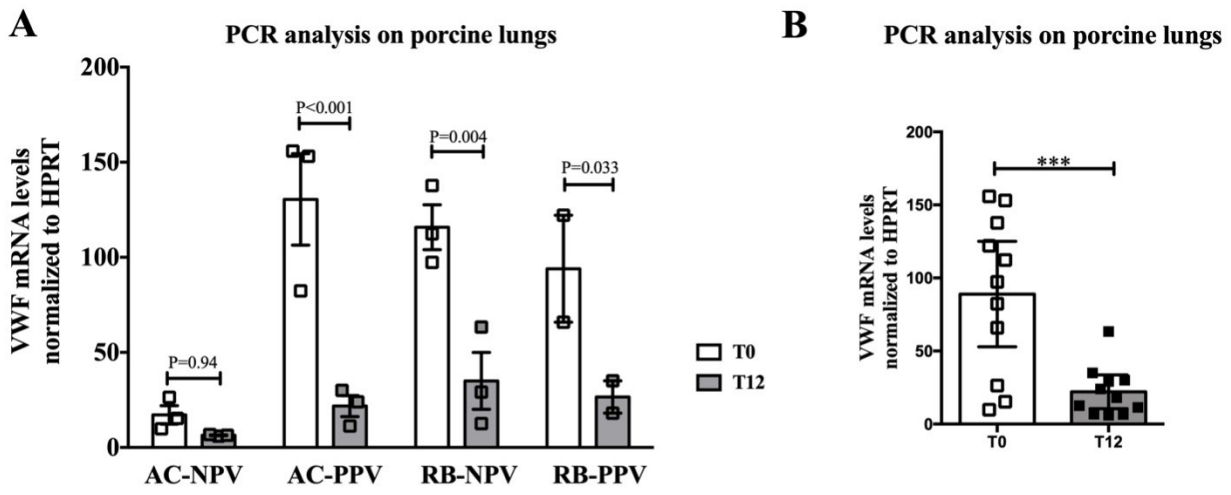

Figure 5.3.4. Quantitative real-time PCR analysis of VWF mRNA in EVLP perfused lungs.

(A) VWF mRNA levels of lungs at T0 and EVLP perfused (T12) for 12 hours under various conditions were determined by qRT-PCR. (B) The total average of VWF mRNA levels at T0 and lungs perfused under EVLP for 12 hours were determined by qRT-PCR. AC-NPV represents negative pressure with acellular perfusate, AC-PPV represents positive pressure with acellular perfusate, RB-NPV represents negative pressure with pRBC perfusate and, RB-PPV represents positive pressure with pRBC perfusate. HPRT mRNA was used as control for normalization. Data are shown as the mean ± SEM. (*$p < 0.05$)

To determine whether alterations of the VWF mRNAs are reflected at the protein levels under different EVLP conditions, Western blot analyses were performed on tissue samples from the four EVLP treated groups. The results demonstrated that consistent with the results of RNA analyses, cellular VWF protein levels were also significantly reduced in lungs, which were

perfused under EVLP for 12 hours compared to control. This effect was highly significant for three of the four EVLP treatment's conditions (Figure 5.3.5).

These results collectively demonstrated that exposure to warm perfusion and reduced hypoxic and cold exposure time significantly reduced the expression levels of VWF in harvested lungs.

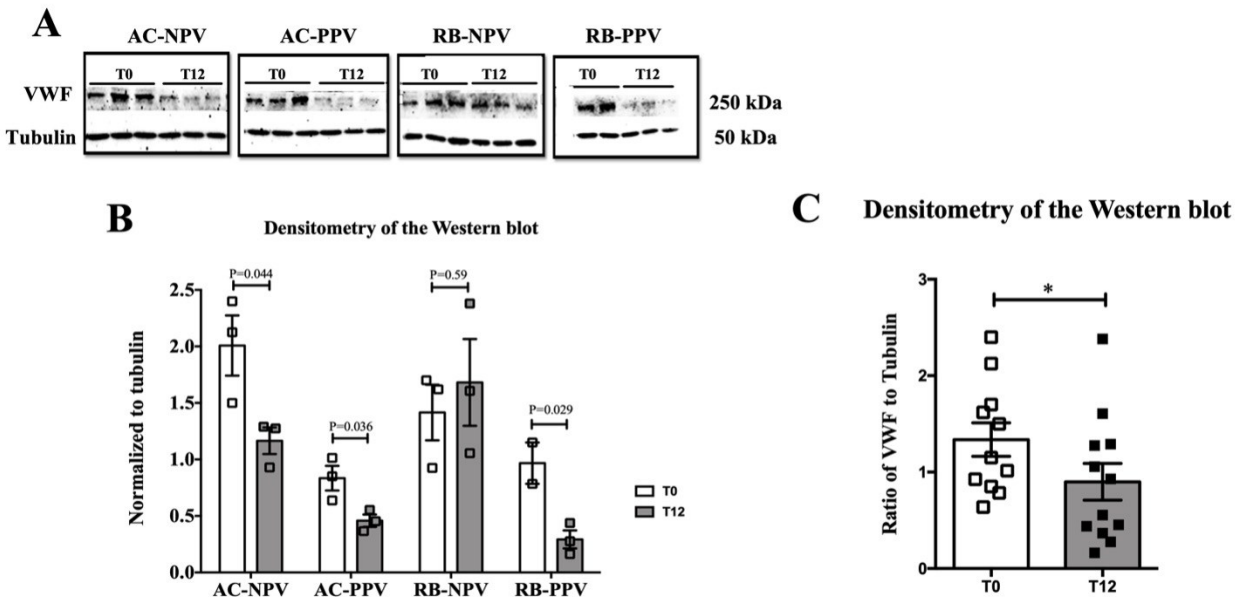

Figure 5.3.5. Analysis of VWF protein expression levels in lung samples under EVLP at different conditions.

(A) Western blot analysis of lung tissues at T0 and after 12 hours EVLP perfusion under various conditions. (AC-NPV) Negative pressure with acellular perfusate, (AC-PPV) positive pressure with acellular perfusate, (RB-NPV) negative pressure with pRBC perfusate and, (RB-PPV) positive pressure with pRBC perfusate. (B) The graphs represent quantitative analysis of VWF signals after normalization to the tubulin. Results of 3 independent porcine (n=3 per group) are shown in all groups except in RB-PPV group at T0 (n=2). (C) The total average of VWF protein levels at T0 and lungs perfused under EVLP for 12 hours was determined by Western blot analysis. Data are shown as the mean ± SEM. (*$p < 0.05$)

5.3.4.4 VWF expression pattern in lungs that are maintained under warm perfusion prior and post transplantation

Since our data demonstrated that SCS preservation altered the VWF expression pattern in the lung microvascular endothelial cells of pig's lungs, thus, we proceeded to determine whether *ex vivo* lung preservation also alters the VWF expression pattern in the endothelial cells of lung. For these analyses, we performed double stained immunofluorescence analyses using CD31 (endothelial cell marker) and VWF antibodies on samples of lungs that were preserved under EVLP and transplanted. These included IV (in vivo pre transplant), PT0 (immediately at the beginning of transplant and after 12 hours at EVLP condition) and PT4 (4 hours post-transplant and after 12 hours maintenance in EVLP) lungs.

The data demonstrated that there was no alteration in VWF expression pattern in vasculature of lung in both PT0 and PT4 compared to IV (Figure 5.3.6 A)**.** Quantitative analysis was performed and reported as a ratio of VWF and CD31 positive sites (indicative of vessels that express VWF) to the total CD31 positive sites (representing total number of vessels) per field of view (Figure 5.3.6). These results demonstrated that preservation of lungs under EVLP conditions prior to transplantation does not alter VWF expression pattern. Majority of VWF expression was detected in large vessels in IV samples, and still after 12 hours preservation under EVLP setting and post transplantation the VWF expression and distribution pattern did not change through vasculature.

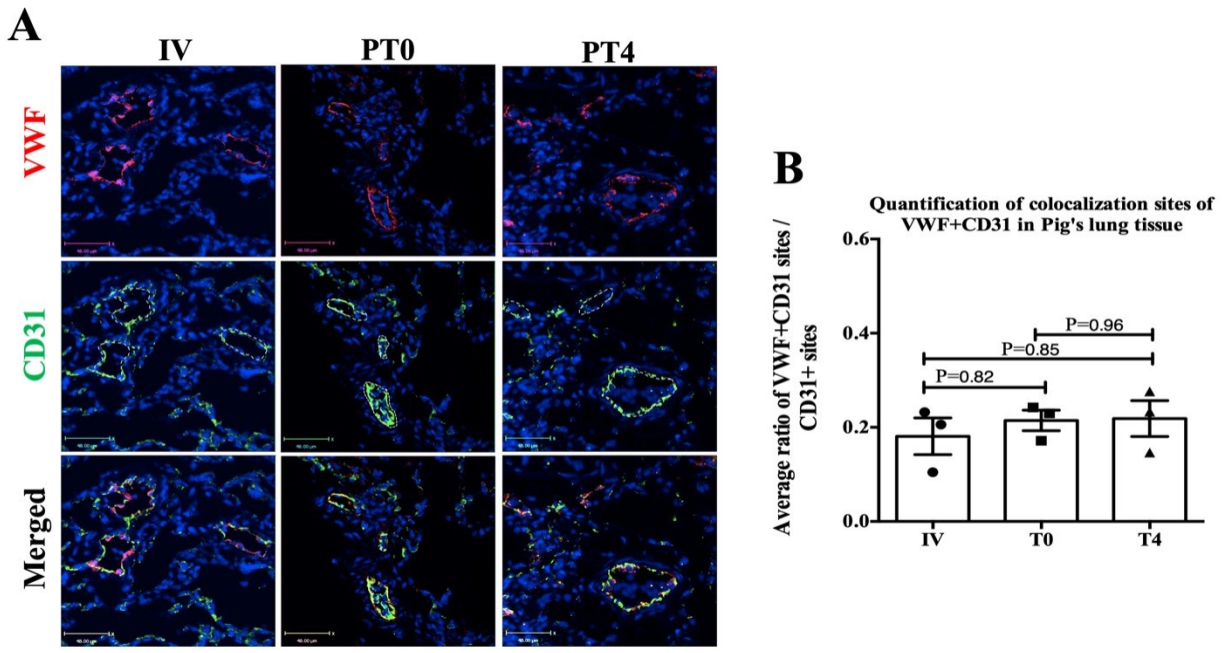

Figure 5.3.6. Immunofluorescent analysis of lungs that were kept under EVLP before and after transplant.

(A) Immunofluorescence analyses were performed using double staining for CD31 (green) and VWF (red) on pig's lung samples as described in Material and methods. (IV: in vivo pretransplant, PT0: immediately at the beginning of transplant and after 12 hours at EVLP, PT4: four hours post transplantation and after 12 hours at EVLP). Representative large/medium vessels are marked with dotted lines in the image. Scale bar is 48 µm. (B) Quantification analysis was performed to identify the ratio of VWF expressing CD31 positive vessels to total CD31 positive vessels based on analyses of 5 fields of view of lung sections from 3 pigs for each group (n=3). Data are shown as the mean ± SEM. (*$p < 0.05$).

5.3.4.5 Limb's blood vessels samples

Since previously we reported that hypoxia regulated VWF expression in an organ specific manner [41], in current study we sought to investigate whether VWF response to organ preservation condition also exhibits organ-specificity. Toward this goal, we used blood vessels isolated from pig's limb, which were maintained under normothermic perfusion (RBC perfusate at 38°C) for 0 or12 hours. The results showed a significant reduction in VWF at both mRNA and protein levels in limb's blood vessels samples, at T12 compared to T0 (Figure 5.3.7)**.** Results for individual samples presented in supplementary data (Figure 5.3.11).

These results suggest that organ preservation methods, which reduces vascular exposure to hypoxic and cold conditions significantly reduces the levels of VWF expression; and that this VWF response is not restricted to lung.

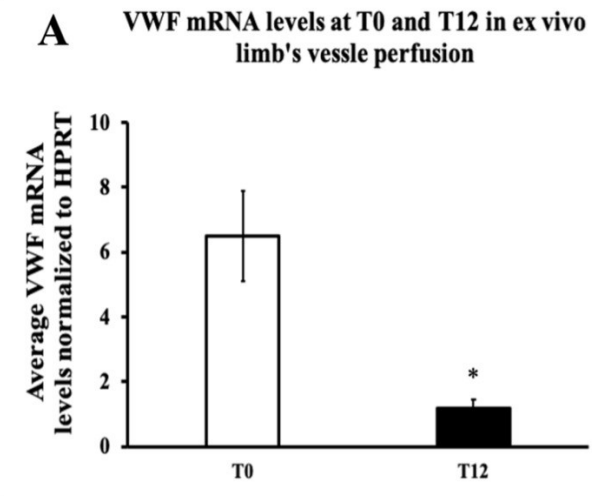

Figure 5.3.7. VWF mRNA detection at T0 and T12 samples from normothermic perfused limbs.

(A) Quantitative real time PCR analysis was done to determine the mRNA levels of VWF, normalized to HPRT, in vessels of limbs at T0 (white bar) and after 12 hours perfusion (T12)

(black bar). Results of 5 independent experiments are shown individually. Error bars are representation of three technical replicates for each sample. (B) Average level of VWF mRNA were reported for the 5 independent experiments (n=5 per group). Data are shown as the mean ± SEM. (*p < 0.05)

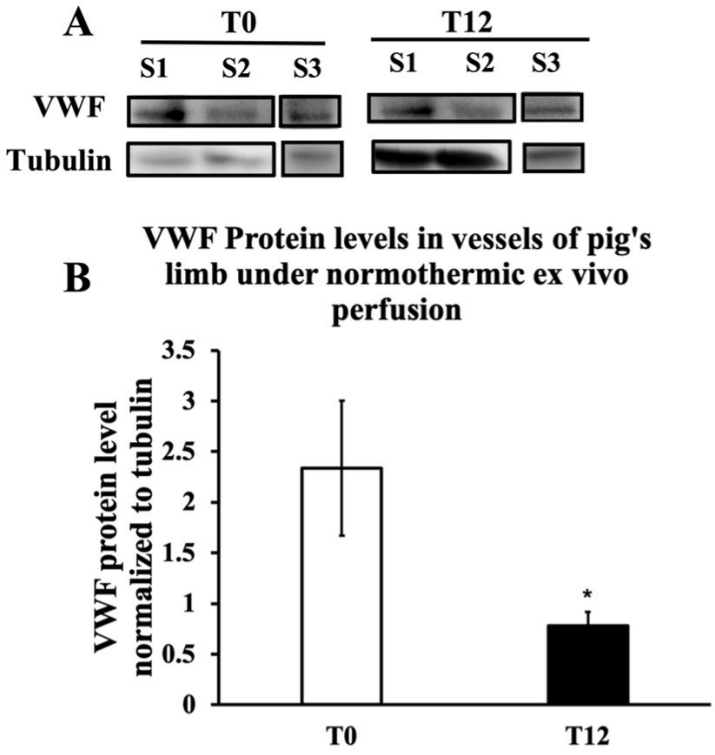

Figure 5.3.8. Immunoblotting analysis to detect VWF protein levels at T0 and T12 vessels samples from perfused limbs.

(A) Western blot analyses to detect VWF protein in *ex vivo* perfused limb's vessels at T0 and T12 time points. Tubulin was used as the loading control. (B) Immunoblotting quantification is reported as a graph after normalization to tubulin from 3 independent experiments (n=3). (S1= Sample#1, S2= Sample #2, S3= Sample #3). Data are shown as the mean ± SEM. (*p < 0.05).

5.3.5 Discussion

Providing the best storage condition for lungs prior to transportation is a vital step to minimize post-transplantation complications and improve graft survival [114]. VWF is known as a key player molecule in platelet aggregate formation and thrombosis. VWF levels and pattern of expression in the lung were shown to be altered in response to hypoxia [41], which is a condition that is normally experienced by organs that are preserved for transplantation.

In the present study, we used two models of organ preservation before transplantation, to investigate the effect of these pre-transplantation conditions on the expression of VWF molecule. These models include the standard method of lung preservation, which is Static Cold Storage (SCS); and the *ex vivo* lung perfusion (EVLP), which is a novel method of preservation that reduces the period of hypoxic and cold exposure conditions.

Our analysis demonstrated that after 12 hours in SCS condition, VWF mRNAs and proteins in lungs' samples showed a significant increase or unaltered levels compared to T0 control groups. Generally, under cold conditions, many cellular processes, including transcription, translation, and metabolic activities, are significantly reduced, resulting in decreased levels of many gene products [409]. Thus, the observation that VWF mRNA and protein levels were not reduced suggests that either the VWF gene is among the few genes that are not the target of cold-induced reduction or hypoxia-induced increase overcomes cold-induced reduction or both.

The hypothesis that VWF expression is not as susceptible to cold-induced reduction as other molecules is supported by the report of Horioka et al. that demonstrated increased VWF expression in cultured splenic sinusoidal endothelial and human umbilical vein endothelial (HUVEC) cells that were exposed to low temperature [410]. However, the cold conditions used for cultured cells were 20 ºC (instead of the usual 37 ºC) for 1 hour, while the SCS conditions for organ storage were

4 ºC for 12 hours, which is expected to reduce all cellular functions significantly. We hypothesize that non-significant increase in some T12 mRNA and protein levels in SCS lungs, may result from opposing effects of general reduction in the rate of transcription/translation and increase in response to hypoxia and cold exposure, leading to dampening the final affect. However, the combined effect of low temperature and hypoxic condition as external stimuli together, which is the case in SCS, has not been fully investigated yet. This combination may trigger unknown inhibitory pathway(s) to resist against high level hypoxia-induced increase in VWF expression. Another potential explanation for non-significant but increasing trends in the protein levels of VWF in SCS samples may relate to VWF cellular storage mechanism. Endothelial cells maintain a significant level of cellular VWF protein in storage organelles known as Weibel–Palade bodies. In response to stimuli, including hypoxia, endothelial cells (in addition to increasing VWF expression levels) undergo degranulation and release VWF from storage.

The storage organelles are subsequently replenished with newly synthesized proteins [47]. Thus, we hypothesize that this process may have masked potential increase in VWF protein levels, which is expected as a consequence of hypoxia/cold exposure. Furthermore, we tested the effect of preservation condition on VWF vascular tree distribution pattern in lungs. Previously the heterogeneity of VWF expression in the pig's lung has been reported. ECs from macro vasculature, including pulmonary artery and veins, strongly express VWF, whereas ECs from microvessels, such as alveolar capillaries, either do not express VWF or express VWF at very low levels [411]. However, in the current study, we demonstrated that while in pre-transplanted lung VWF expressions were barely detectable in a few small vessels, in SCS-maintained transplanted lungs, the number of micro vasculatures that expressed VWF significantly increased. This result was consistent with our previous study, where we demonstrated that hypoxia exposure activated de

novo VWF expression in the small vessels of the lung. These data suggest potential increase in VWF at both mRNA and protein levels, as well as de novo expression in micro vessels, in SCS-preserved samples. This may, consequently, raise the risk of thrombus formation and organ dysfunction after transplantation in the recipient body. On the other hand, *ex vivo* lung perfusion (EVLP) provides nutrition and oxygen circulating through the organ (similar to physiological condition) outside of the body. Data showed significant reduction in VWF mRNA and protein levels in different EVLP groups. Previously, our group has shown that lung oxygenation during EVLP improved with cellular perfusate but did not alter significantly between two different ventilation techniques (PPV or NPV) during EVLP [382]. Our data suggest that as long as enough oxygen is provided to the organs by EVLP setting, irrespective of ventilation type, this procedure is highly effective in reducing the expression of the procoagulant molecule, VWF. The observation that expression of VWF during EVLP not only did not remain steady but, in fact was significantly reduced is a noteworthy observation and needs to be further studied. However, it could be a potential way to reduce the risk of future thrombotic complications in transplanted organs.

IF analysis demonstrated that transplanted lungs maintained under EVLP exhibited VWF expression mainly in the endothelial cells of large/medium vessels, resembling an in vivo (pre-transplant) sample. This result suggests that EVLP not only reduces the level of VWF expression in the lungs but also preserves the distribution pattern of VWF in large vessels, a feature not observed in transplanted lungs preserved through SCS. Previously, we reported that endothelial cells of different organs exhibit heterogeneity in their response to hypoxia with regard to VWF expression [41]. Thus, we suggest determining whether the EVLP method that dampens hypoxia-induced VWF upregulation is also observed in other organs in addition to the lung. Limbs from pigs which were similarly maintained under EVLP condition exhibited similar responses with

regard to VWF demonstrating that the effect is not restricted to the lung and may be applicable to multiple organs. Reduction of VWF expression through *ex vivo* normothermic perfusion may has a significant impact on reducing potential thrombogenic complications. However, it should be noted that thrombus formation is a complex process that includes other factors in addition to VWF. For instance, fibrin deposition and/or catalytic molecules, including VWF cleaving enzymes, could also be altered in response to hypoxia and consequently affect the process of thrombus formation. Nevertheless, modulation of VWF response, as a primary initiator of thrombosis, may be highly effective in reducing thrombotic complications.

5.3.6 Supplementary data

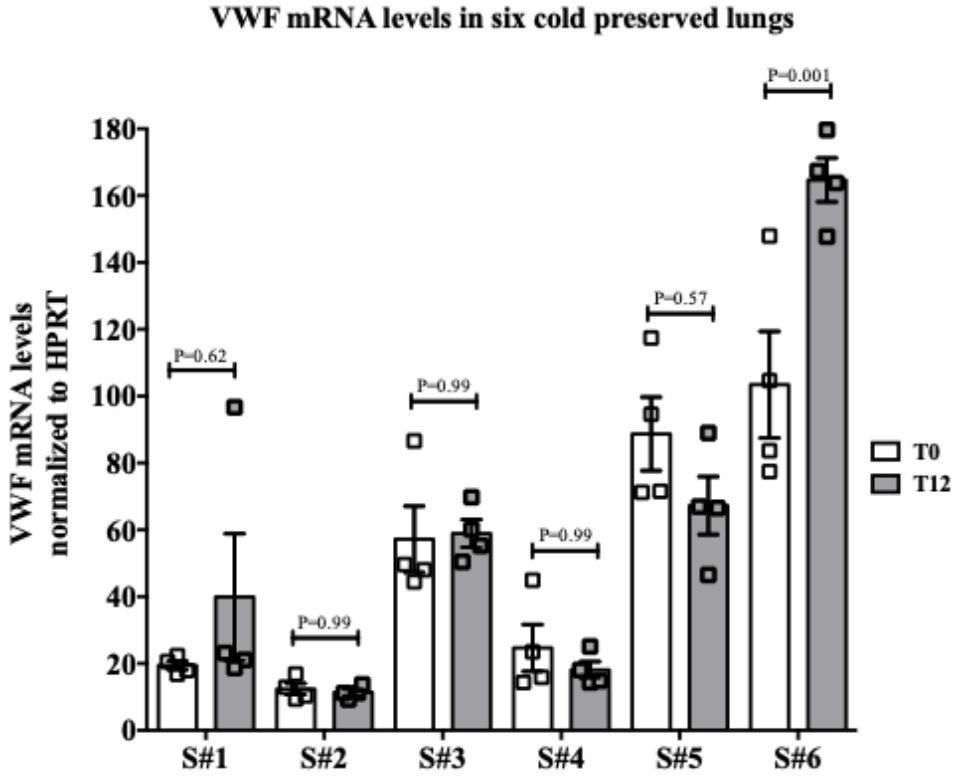

Figure 5.3.9. Analysis of VWF mRNA levels at T0 and T12 from SCS preserved lungs in individual porcine.

Quantitative real time PCR analysis was used to determine the mRNA levels of VWF, normalized to HPRT mRNA, in lungs samples at T0 (white bar) and after 12 hours in SCS (T12) (black bar). Average levels of VWF mRNA were reported for the 6 individual animal (n=4, technical replicate). Data are shown as the mean ± SEM. (*$p < 0.05$)

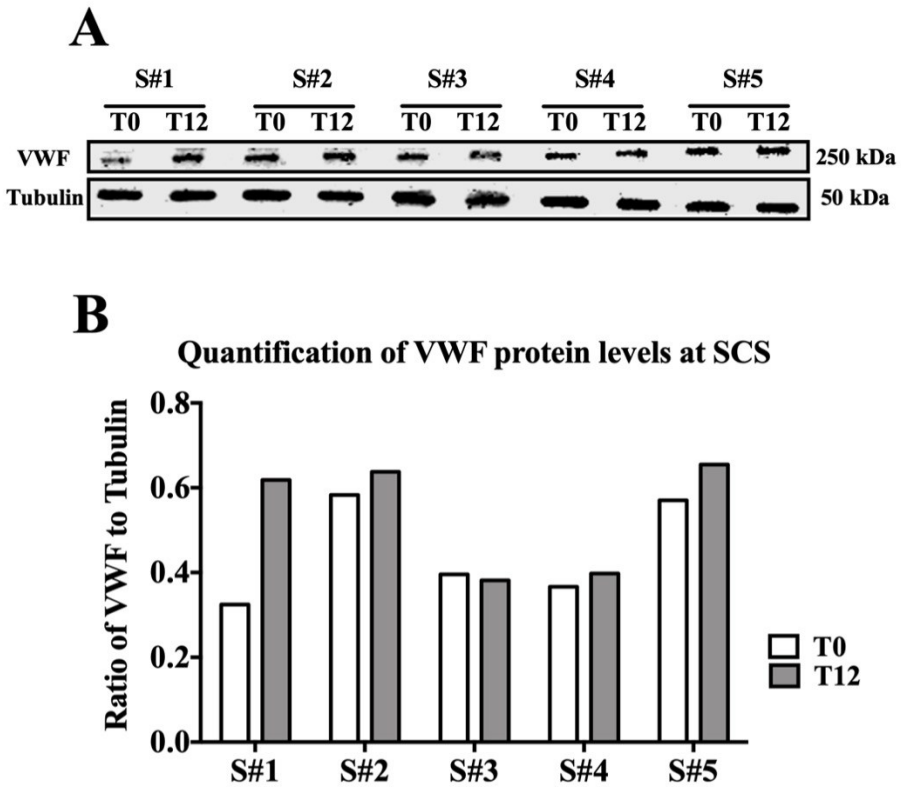

Figure 5.3.10. Analysis of VWF protein levels at T0 and T12 from SCS preserved lungs.

(A) Western blot analyses were performed to detect VWF protein in preserved lungs at T0 and in SCS condition for 12 hours (T12). Tubulin was used as normalization control. (B) The graphs represent densitometry quantification after normalization to tubulin for individual animal (n=5 per group). Data are shown as the mean ± SEM. (*$p < 0.05$)

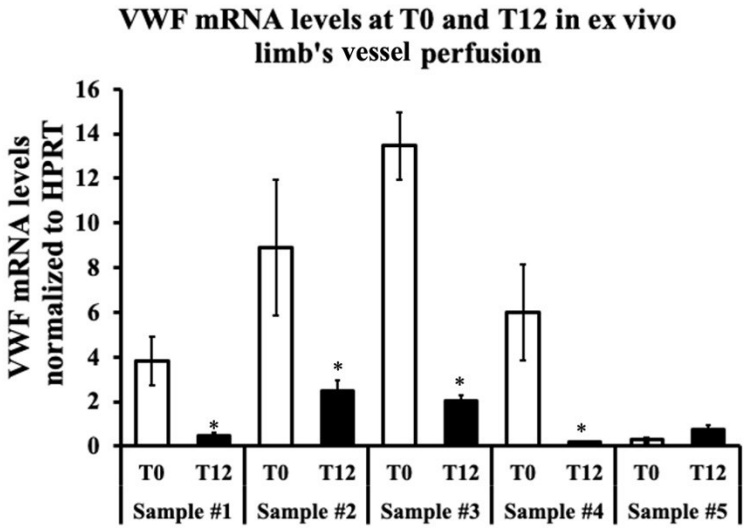

Figure 5.3.11. Analysis of VWF mRNA levels at T0 and T12 from *ex vivo* limb's blood vessels preserved in individual porcine.

Quantitative real time PCR analysis was done to determine the mRNA levels of VWF, normalized to HPRT, in vessels of limbs at T0 (white bar) and after 12 hours perfusion (T12) (black bar). Data from each animal are shown individually. Error bars are representation of technical replicates for each sample.

6 Chapter 6

6.1 Summary

In the current thesis, I investigated whether aging, and organ transplants provide signals or stimuli that trigger VWF upregulation. In exploring the mechanism and consequences of VWF response to aging, I used both in vivo and in vitro aging models. As an in vivo model, I used 3-month-old and 18-19-month-old mice as a young and aged group, respectively. For in vitro model I used HUVECs, which were kept in culture for three weeks without passaging (HUVEC W3) compared to those maintained in culture for 3 days (HUVEC W0).

My analyses demonstrated that:

I) VWF levels and activity rise with age. The ELISA and multimer analysis of the young and aged mice plasma indicated that circulating functional large VWF multimers are elevated in aged mice.

II) An organ-specific aged-related increase in VWF transcription and translation was demonstrated. VWF upregulates at both mRNA and protein levels in the brain, liver, and lung but not the heart and kidney in aged mice compared to young mice.

III) The study demonstrated that aging prompts increasing microvascular endothelial cells in the liver, lung, and brain to express VWF. This procoagulant shift in microvascular endothelial cells could enhance the thrombotic properties of these endothelial cells in the vascular bed.

IV) Age-associated increased VWF expression was accompanied by increased platelet aggregate formation in aged organ vasculature compared to young.

V) In brain, which is one of the organs that demonstrated age-associated increase in VWF, senescent markers including SA-β-gal and p53 were detected in increasing proportion of VWF expressing endothelial cells.

VI) Knocking down p53 by using specific SiRNA eliminated the senescent-induced VWF upregulation in HUVECs.

In exploring whether transplant procedure is correlated with VWF upregulation in donor organ, I explored whether organ preservation procedures can affect VWF production. As previously discussed, the original idea for this study stemmed from our group's earlier investigation of VWF upregulation in response to hypoxia. Specifically, the observation that in lung, hypoxia leads to redistribution of VWF expression pattern, from primarily large vessels to increasing proportion of microvasculature, prompted the choice of lung transplant for this study. In the transplant process, the organs, including lung, which are going to be transplanted undergo exposure to hypoxia, thus we explored whether procedures that have differing exposure to hypoxia may have distinct effects on VWF response in lung transplant procedure.

My analyses demonstrated that:

I) Under the static cold storage condition (SCS), VWF mRNA and protein levels in lung samples remained unchanged compared to the control group.

II) In SCS-maintained lungs, there was an alteration in VWF expression pattern, similar to that observed in hypoxia-exposed mice, a significant increase in microvasculatures expressing VWF following transplantation was observed.

III) There were significant decreases in VWF mRNA and protein levels in lungs across various ex vivo lung perfusion (EVLP) groups, which have a significantly reduced hypoxia and hypothermic exposure period. Regardless of the type of ventilation or perfusate used, this method effectively reduced VWF expression.

IV) The EVLP technique did not alter the von Willebrand factor distribution pattern within the lungs' vasculature after transplantation.

V) The EVLP technique, which mitigates the hypoxia-induced increase in von Willebrand factor, is not restricted to the lungs; it is also observed in other organs. I demonstrated that VWF reduction at both mRNA and protein levels occurs similarly in the endothelial cells of pig's limb blood vessels. This finding suggests that the benefit of EVLP regarding VWF expression reduction could extend and apply to multiple organs.

6.2 General discussion and future directions

Unregulated VWF upregulation may arise as a result of physiological processes leading to thrombotic complications. We explored aging and transplant procedures as two such processes and demonstrated that aging in healthy population, and transplant procedures that exposes lung to extended hypoxia, upregulate VWF expression. My analyses of age-associated VWF upregulation demonstrated that even under normal physiological aging, a pathophysiological process, namely acquiring senescence phenotype is the major contributing factor to upregulation of VWF. The significant reduction in VWF and platelets aggregate formation by senolytic drug treatment

demonstrated that this process is a target for potential effective therapeutic approaches for intervention. Also, with regard to transplant induced VWF upregulation, results demonstrating that warm perfusion disrupts the upregulation and microvascular expression of VWF in lung, strongly suggests that this process may be a target of effective intervention towards combating transplant associated thrombotic complications. These findings offer a compelling anti-thrombotic advantage in organ transplantation, requiring further investigation. The results of aging and transplant-related VWF analyses also underscore the organ-specific and endothelial cell heterogeneity of responses to conditions that promote VWF upregulation. My studies, in exploring the mechanism and consequences of VWF upregulation, address a knowledge gap and provide insights toward developing targeted therapies and interventions for combating VWF-associated thrombotic complications.

Future plans for this project include:

1. Investigating the association of VWF expression with thrombogenicity in vasculatures of aged organs in greater detail by determining the proportion of platelet aggregates that are stable and contain fibrin deposits, compared to those without, and hence exhibiting reversible characteristics.
2. Determining whether VWF is necessary and sufficient for age-associated platelet aggregates formation in target organs, by using VWF knockout and determining the prevalence of platelets aggregate formation in the vasculature of these mice when aged.
3. Determine the association of VWF-mediated platelet aggregates formation with organ dysfunction, specifically brain function by exploring the cognitive behaviour in aged mice that are either VWF KO, or wild type but treated with senolytic drug.

4. Determine the molecular basis of senescent associated VWF upregulation by further exploring the role of p53, and pattern of previously identified transcription factor associations with the VWF promoter and its chromatin modifications.
5. Determine whether transplant associated VWF upregulation correlates with increased functional VWF by assessing intracellular VWF multimer size in SCS-maintained and EVLP-preserved porcine before and after transplantation.
6. Explore whether transplant-induced VWF upregulation exhibit organ-specific characteristics that were observed in hypoxia-induced VWF upregulation, by determining VWF expression levels and patterns in heart and kidney transplantation procedure, as performed for lung.

   The collected data will be valuable for designing effective anti-thrombotic approaches specific to vascular beds and in response to aging/senescence and organ transplant procedures.

References

1. Von Willebrand, E., Hereditary pseudohaemophilia. Haemophilia: the official journal of the World Federation of Hemophilia, 1999. 5(3): p. 223-222.
2. Swami, A. and V. Kaur, von Willebrand disease: a concise review and update for the practicing physician. Clinical and applied thrombosis/hemostasis, 2017. 23(8): p. 900-910.
3. EA, W., Hereditar pseudohemofili. Finska Lakarsallskapets Hand, 1926. 1(67): p. 7-112.
4. Sadler, J.E., Biochemistry and genetics of von Willebrand factor. Annual review of biochemistry, 1998. 67(1): p. 395-424.
5. Sadler, J.E., et al., Cloning and characterization of two cDNAs coding for human von Willebrand factor. Proceedings of the National Academy of Sciences, 1985. 82(19): p. 6394-6398.
6. Ginsburg, D., et al., Human von Willebrand factor (vWF): isolation of complementary DNA (cDNA) clones and chromosomal localization. Science, 1985. 228(4706): p. 1401-1406.
7. Lynch, D.C., et al., Molecular cloning of cDNA for human von Willebrand factor: authentication by a new method. Cell, 1985. 41(1): p. 49-56.
8. Verweij, C.L., et al., Construction of cDNA coding for human von Willebrand factor using antibody probes for colony-screening and mapping of the chromosomal gene. Nucleic acids research, 1985. 13(13): p. 4699-4717.
9. Avdonin, P., et al., Von Willebrand Factor in Health and Disease. Biochemistry (Moscow), Supplement Series A: Membrane and Cell Biology, 2021. 15(3): p. 201-218.
10. Palta, S., R. Saroa, and A. Palta, Overview of the coagulation system. Indian journal of anaesthesia, 2014. 58(5): p. 515-523.
11. Bryckaert, M., et al., Of von Willebrand factor and platelets. Cellular and Molecular Life Sciences, 2015. 72(2): p. 307-326.
12. Reininger, A.J., Function of von Willebrand factor in haemostasis and thrombosis. Haemophilia, 2008. 14: p. 11-26.
13. Chauhan, A.K., et al., von Willebrand factor and factor VIII are independently required to form stable occlusive thrombi in injured veins. Blood, 2007. 109(6): p. 2424-2429.
14. Wagner, D.D., Cell biology of von Willebrand factor. Annual review of cell biology, 1990. 6(1): p. 217-242.
15. Patmore, S., S.P.S. Dhami, and J.M. O'Sullivan, Von Willebrand factor and cancer; metastasis and coagulopathies. Journal of Thrombosis and Haemostasis, 2020. 18(10): p. 2444-2456.
16. Franchini, M., et al., von Willebrand factor and cancer: a renewed interest. Thrombosis Research, 2013. 131(4): p. 290-292.
17. Gragnano, F., et al., The role of von Willebrand factor in vascular inflammation: from pathogenesis to targeted therapy. Mediators of inflammation, 2017. 2017.
18. Starke, R.D., et al., Endothelial von Willebrand factor regulates angiogenesis. Blood, The Journal of the American Society of Hematology, 2011. 117(3): p. 1071-1080.
19. Bonthron, D., et al., Nucleotide sequence of pre-pro-von Willebrand factor cDNA. Nucleic acids research, 1986. 14(17): p. 7125.
20. Shelton-Inloes, B.B., et al., Evolution of human von Willebrand factor: cDNA sequence polymorphisms, repeated domains, and relationship to von Willebrand antigen II. Biochemical and biophysical research communications, 1987. 144(2): p. 657-665.

21. de Wit, T.R. and J.A. van Mourik, Biosynthesis, processing and secretion of von Willebrand factor: biological implications. Best Practice & Research Clinical Haematology, 2001. 14(2): p. 241-255.
22. Lenting, P.J., O.D. Christophe, and C.V. Denis, von Willebrand factor biosynthesis, secretion, and clearance: connecting the far ends. Blood, The Journal of the American Society of Hematology, 2015. 125(13): p. 2019-2028.
23. Lancellotti, S., et al., Mechanochemistry of von Willebrand factor. Biomolecular concepts, 2019. 10(1): p. 194-208.
24. Risser, F., et al., Engineered molecular therapeutics targeting fibrin and the coagulation system: a biophysical perspective. Biophysical reviews, 2022. 14(2): p. 427-461.
25. Peyvandi, F., I. Garagiola, and L. Baronciani, Role of von Willebrand factor in the haemostasis. Blood Transfusion, 2011. 9(Suppl 2): p. s3.
26. Sadler, J., von Willebrand factor assembly and secretion. Journal of Thrombosis and Haemostasis, 2009. 7: p. 24-27.
27. Wagner, D.D. and V.J. Marder, Biosynthesis of von Willebrand protein by human endothelial cells: processing steps and their intracellular localization. The Journal of cell biology, 1984. 99(6): p. 2123-2130.
28. Wagner, D.D., T. Mayadas, and V.J. Marder, Initial glycosylation and acidic pH in the Golgi apparatus are required for multimerization of von Willebrand factor. The Journal of cell biology, 1986. 102(4): p. 1320-1324.
29. Keeney, S. and A. Cumming, The molecular biology of von Willebrand disease. Clinical & Laboratory Haematology, 2001. 23(4): p. 209-230.
30. Rehemtulla, A. and R.J. Kaufman, Preferred sequence requirements for cleavage of pro-von Willebrand factor by propeptide-processing enzymes. 1992.
31. Rosnoblet, C., et al., Regulated von Willebrand factor (vWf) secretion is restored by pro-vWf expression in a transfectable endothelial cell line. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 2000. 1495(1): p. 112-119.
32. Haberichter, S.L., von Willebrand factor propeptide: biology and clinical utility. Blood, The Journal of the American Society of Hematology, 2015. 126(15): p. 1753-1761.
33. Ruggeri, Z., Von Willebrand factor, platelets and endothelial cell interactions. Journal of thrombosis and haemostasis, 2003. 1(7): p. 1335-1342.
34. Lopes da Silva, M. and D.F. Cutler, von Willebrand factor multimerization and the polarity of secretory pathways in endothelial cells. Blood, The Journal of the American Society of Hematology, 2016. 128(2): p. 277-285.
35. Stockschlaeder, M., R. Schneppenheim, and U. Budde, Update on von Willebrand factor multimers: focus on high-molecular-weight multimers and their role in hemostasis. Blood Coagulation & Fibrinolysis, 2014. 25(3): p. 206-216.
36. Sporn, L.A., V.J. Marder, and D.D. Wagner, Inducible secretion of large, biologically potent von Willebrand factor multimers. Cell, 1986. 46(2): p. 185-190.
37. Tsai, H.M., et al., The high molecular weight form of endothelial cell von Willebrand factor is released by the regulated pathway. British journal of haematology, 1991. 79(2): p. 239-245.
38. Giblin, J.P., L.J. Hewlett, and M.J. Hannah, Basal secretion of von Willebrand factor from human endothelial cells. Blood, The Journal of the American Society of Hematology, 2008. 112(4): p. 957-964.

39. Dmitrieva, N.I. and M.B. Burg, Secretion of von Willebrand factor by endothelial cells links sodium to hypercoagulability and thrombosis. Proceedings of the National Academy of Sciences, 2014. 111(17): p. 6485-6490.
40. Xiang, Y., et al., Hyperglycemia repression of miR-24 coordinately upregulates endothelial cell expression and secretion of von Willebrand factor. Blood, The Journal of the American Society of Hematology, 2015. 125(22): p. 3377-3387.
41. Mojiri, A., et al., Hypoxia results in upregulation and de novo activation of von Willebrand factor expression in lung endothelial cells. Arteriosclerosis, thrombosis, and vascular biology, 2013. 33(6): p. 1329-1338.
42. Jahroudi, N., A.M. Ardekani, and J.S. Greenberger, Ionizing irradiation increases transcription of the von Willebrand factor gene in endothelial cells. 1996.
43. Mussbacher, M., et al., Cell type-specific roles of NF-κB linking inflammation and thrombosis. Front Immunol. 2019; 10: 85. 2019.
44. Vischer, U.M. and C.B. Wollheinn, Epinephrine induces von Willebrand factor release from cultured endothelial cells: involvement of cyclic AMP-dependent signalling in exocytosis. Thrombosis and haemostasis, 1997. 77(06): p. 1182-1188.
45. Kaufmann, J.E., et al., Vasopressin-induced von Willebrand factor secretion from endothelial cells involves V2 receptors and cAMP. The Journal of clinical investigation, 2000. 106(1): p. 107-116.
46. Xiong, Y., et al., Vascular endothelial growth factor (VEGF) receptor-2 tyrosine 1175 signaling controls VEGF-induced von Willebrand factor release from endothelial cells via phospholipase C-γ1-and protein kinase A-dependent pathways. Journal of biological chemistry, 2009. 284(35): p. 23217-23224.
47. Schillemans, M., et al., Exocytosis of Weibel–Palade bodies: how to unpack a vascular emergency kit. Journal of Thrombosis and Haemostasis, 2019. 17(1): p. 6-18.
48. Galbusera, M., et al., Fluid shear stress modulates von Willebrand factor release from human vascular endothelium. Blood, The Journal of the American Society of Hematology, 1997. 90(4): p. 1558-1564.
49. Franchini, M., et al., Relationship between ABO blood group and von Willebrand factor levels: from biology to clinical implications. Thrombosis journal, 2007. 5: p. 1-5.
50. Gallinaro, L., et al., A shorter von Willebrand factor survival in O blood group subjects explains how ABO determinants influence plasma von Willebrand factor. Blood, The Journal of the American Society of Hematology, 2008. 111(7): p. 3540-3545.
51. Ward, S.E., J.M. O'Sullivan, and J.S. O'Donnell, The relationship between ABO blood group, von Willebrand factor, and primary hemostasis. Blood, The Journal of the American Society of Hematology, 2020. 136(25): p. 2864-2874.
52. Souto, J.C., et al., Functional effects of the ABO locus polymorphism on plasma levels of von Willebrand factor, factor VIII, and activated partial thromboplastin time. Arteriosclerosis, thrombosis, and vascular biology, 2000. 20(8): p. 2024-2028.
53. Gill, J.C., et al., The effect of ABO blood group on the diagnosis of von Willebrand disease. 1987.
54. Michels, A., D. Lillicrap, and M. Yacob, Role of von Willebrand factor in venous thromboembolic disease. JVS-Vascular Science, 2022. 3: p. 17-29.
55. Zheng, X.L., ADAMTS13 and von Willebrand factor in thrombotic thrombocytopenic purpura. Annual review of medicine, 2015. 66: p. 211-225.

56. Ruggeri, Z.M. and T.S. Zimmerman, von Willebrand factor and von Willebrand disease [published erratum appears in Blood 1988 Mar; 71 (3): 830]. 1987.
57. Sadler, J.E., A revised classification of von Willebrand disease. Thrombosis and haemostasis, 1994. 71(04): p. 520-525.
58. Vischer, U., von Willebrand factor, endothelial dysfunction, and cardiovascular disease. Journal of thrombosis and haemostasis, 2006. 4(6): p. 1186-1193.
59. Berber, E., The molecular genetics of von Willebrand disease. Turkish Journal of Haematology, 2012. 29(4): p. 313.
60. Lillicrap, D. The molecular genetics of von Willebrand disease. in Hematology Meeting Reports (formerly Haematologica Reports). 2005.
61. Bharati, K.P. and U.R. Prashanth, Von Willebrand disease: an overview. Indian journal of pharmaceutical sciences, 2011. 73(1): p. 7.
62. Sadler, J., et al., Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. Journal of thrombosis and haemostasis, 2006. 4(10): p. 2103-2114.
63. Slayton, W.B., et al., Type 2B von Willebrand disease associated with the release of platelet agglutinates from megakaryocytes in the bone marrow. Journal of pediatric hematology/oncology, 2008. 30(9): p. 708-711.
64. Ruggeri, Z.M., et al., Heightened interaction between platelets and factor VIII/von Willebrand factor in a new subtype of von Willebrand's disease. New England Journal of Medicine, 1980. 302(19): p. 1047-1051.
65. Castaman, G., et al., Von Willebrand's disease in the year 2003: towards the complete identification of gene defects for correct diagnosis and treatment. Haematologica, 2003. 88(1): p. 94-108.
66. Tischer, A., et al., Misfolding of vWF to pathologically disordered conformations impacts the severity of von Willebrand disease. Biophysical journal, 2014. 107(5): p. 1185-1195.
67. Keesler, D.A. and V.H. Flood, Current issues in diagnosis and treatment of von Willebrand disease. Research and Practice in Thrombosis and Haemostasis, 2018. 2(1): p. 34-41.
68. Ruggeri, Z.M., The role of von Willebrand factor in thrombus formation. Thrombosis research, 2007. 120: p. S5-S9.
69. Yau, J.W., H. Teoh, and S. Verma, Endothelial cell control of thrombosis. BMC cardiovascular disorders, 2015. 15: p. 1-11.
70. Denorme, F., K. Vanhoorelbeke, and S.F. De Meyer, von Willebrand factor and platelet glycoprotein Ib: a thromboinflammatory axis in stroke. Frontiers in immunology, 2019. 10: p. 495067.
71. Schneider, M.F., et al., Platelet adhesion and aggregate formation controlled by immobilised and soluble VWF. BMC molecular and cell biology, 2020. 21: p. 1-13.
72. Mohri, H., et al., Isolation of the von Willebrand factor domain interacting with platelet glycoprotein Ib, heparin, and collagen and characterization of its three distinct functional sites. Journal of Biological Chemistry, 1989. 264(29): p. 17361-17367.
73. De Marco, L., et al., von Willebrand factor interaction with the glycoprotein IIb/IIa complex. Its role in platelet function as demonstrated in patients with congenital afibrinogenemia. The Journal of clinical investigation, 1986. 77(4): p. 1272-1277.
74. Ruggeri, Z.M., Platelets in atherothrombosis. Nature medicine, 2002. 8(11): p. 1227-1234.

75. Orstavik, K., et al., Factor VIII and factor IX in a twin population. Evidence for a major effect of ABO locus on factor VIII level. American journal of human genetics, 1985. 37(1): p. 89.
76. de Lange, M., et al., The genetics of haemostasis: a twin study. The Lancet, 2001. 357(9250): p. 101-105.
77. Souto, J.C., et al., Genome-wide linkage analysis of von Willebrand factor plasma levels: results from the GAIT project. Thrombosis and haemostasis, 2003. 89(03): p. 468-474.
78. Campos, M., et al., Genetic determinants of plasma von Willebrand factor antigen levels: a target gene SNP and haplotype analysis of ARIC cohort. Blood, The Journal of the American Society of Hematology, 2011. 117(19): p. 5224-5230.
79. Keightley, A.M., et al., Variation at the von Willebrand factor (vWF) gene locus is associated with plasma vWF: Ag levels: identification of three novel single nucleotide polymorphisms in the vWF gene promoter. Blood, The Journal of the American Society of Hematology, 1999. 93(12): p. 4277-4283.
80. Smith, N.L., et al., Novel associations of multiple genetic loci with plasma levels of factor VII, factor VIII, and von Willebrand factor: The CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) Consortium. Circulation, 2010. 121(12): p. 1382-1392.
81. Swystun, L.L. and D. Lillicrap, Genetic regulation of plasma von Willebrand factor levels in health and disease. Journal of Thrombosis and Haemostasis, 2018. 16(12): p. 2375-2390.
82. Smith, N.L., et al., Genetic variation associated with plasma von Willebrand factor levels and the risk of incident venous thrombosis. Blood, The Journal of the American Society of Hematology, 2011. 117(22): p. 6007-6011.
83. Ye, S., et al., Platelet secretion and hemostasis require syntaxin-binding protein STXBP5. The Journal of clinical investigation, 2014. 124(10): p. 4517-4528.
84. Zhu, Q., et al., Syntaxin-binding protein STXBP5 inhibits endothelial exocytosis and promotes platelet secretion. The Journal of clinical investigation, 2014. 124(10): p. 4503-4516.
85. Ogiwara, K., et al. Scavenger receptor class A member 5 (SCARA5) binds and internalizes VWF in vitro: a novel candidate VWF clearance receptor. in JOURNAL OF THROMBOSIS AND HAEMOSTASIS. 2015. WILEY-BLACKWELL 111 RIVER ST, HOBOKEN 07030-5774, NJ USA.
86. Swystun, L.L., et al., The endothelial cell receptor stabilin-2 regulates VWF-FVIII complex half-life and immunogenicity. The Journal of clinical investigation, 2018. 128(9): p. 4057-4073.
87. Sabater-Lleal, M., et al., Genome-wide association transethnic meta-analyses identifies novel associations regulating coagulation factor VIII and von Willebrand factor plasma levels. Circulation, 2019. 139(5): p. 620-635.
88. Mancuso, D., et al., Structure of the gene for human von Willebrand factor. Journal of Biological Chemistry, 1989. 264(33): p. 19514-19527.
89. Jahroudi, N. and D.C. Lynch, Endothelial-cell-specific regulation of von Willebrand factor gene expression. Molecular and cellular biology, 1994. 14(2): p. 999-1008.
90. Peng, Y. and N. Jahroudi, The NFY transcription factor functions as a repressor and activator of the von Willebrand factor promoter. Blood, The Journal of the American Society of Hematology, 2002. 99(7): p. 2408-2417.

91. Peng, Y. and N. Jahroudi, The NFY transcription factor inhibits von Willebrand factor promoter activation in non-endothelial cells through recruitment of histone deacetylases. Journal of Biological Chemistry, 2003. 278(10): p. 8385-8394.
92. Wang, X., et al., Histone H1–like protein participates in endothelial cell–specific activation of the von Willebrand factor promoter. Blood, 2004. 104(6): p. 1725-1732.
93. Jahroudi, N., A.M. Ardekani, and J.S. Greenberger, An NF1-like protein functions as a repressor of the von Willebrand factor promoter. Journal of Biological Chemistry, 1996. 271(35): p. 21413-21421.
94. Hough, C., et al., Cell type–specific regulation of von Willebrand factor expression by the E4BP4 transcriptional repressor. Blood, 2005. 105(4): p. 1531-1539.
95. Schwachtgen, J.-L., et al., Ets transcription factors bind and transactivate the core promoter of the von Willebrand factor gene. Oncogene, 1997. 15(25): p. 3091-3102.
96. Schwachtgen, J.-L., et al., Oct-1 is involved in the transcriptional repression of the von willebrand factor gene promoter. Blood, The Journal of the American Society of Hematology, 1998. 92(4): p. 1247-1258.
97. Aird, W.C., et al., Human von Willebrand factor gene sequences target expression to a subpopulation of endothelial cells in transgenic mice. Proceedings of the National Academy of Sciences, 1995. 92(10): p. 4567-4571.
98. Jahroudi, N., et al., Von Willebrand factor promoter targets the expression of amyloid β protein precursor to brain vascular endothelial cells of transgenic mice. Journal of Alzheimer's Disease, 2003. 5(2): p. 149-158.
99. Aird, W.C., et al., Vascular bed–specific expression of an endothelial cell gene is programmed by the tissue microenvironment. The Journal of cell biology, 1997. 138(5): p. 1117-1124.
100. Kleinschmidt, A.M., et al., Sequences in intron 51 of the von Willebrand factor gene target promoter activation to a subset of lung endothelial cells in transgenic mice. Journal of biological chemistry, 2008. 283(5): p. 2741-2750.
101. Nassiri, M., et al., Repressors NFI and NFY participate in organ-specific regulation of von Willebrand factor promoter activity in transgenic mice. Arteriosclerosis, thrombosis, and vascular biology, 2010. 30(7): p. 1423-1429.
102. Borchiellini, A., et al., Quantitative analysis of von Willebrand factor propeptide release in vivo: effect of experimental endotoxemia and administration of 1-deamino-8-D-arginine vasopressin in humans. 1996.
103. Bernardo, A., et al., Effects of inflammatory cytokines on the release and cleavage of the endothelial cell–derived ultralarge von Willebrand factor multimers under flow. Blood, 2004. 104(1): p. 100-106.
104. Li, Y., et al., Plasma von Willebrand factor level is transiently elevated in a rat model of acute myocardial infarction. Experimental and therapeutic medicine, 2015. 10(5): p. 1743-1749.
105. Bertagna, A. and N. Jahroudi, The NFY transcription factor mediates induction of the von Willebrand factor promoter by irradiation. Thrombosis and haemostasis, 2001. 85(05): p. 837-844.
106. Peng, Y., et al., Irradiation modulates association of NF-Y with histone-modifying cofactors PCAF and HDAC. Oncogene, 2007. 26(54): p. 7576-7583.
107. Maniatis, N.A., et al., Endothelial pathomechanisms in acute lung injury. Vascular pharmacology, 2008. 49(4-6): p. 119-133.

108. Sabharwal, A.K., et al., Tissue factor pathway inhibitor and von Willebrand factor antigen levels in adult respiratory distress syndrome and in a primate model of sepsis. American journal of respiratory and critical care medicine, 1995. 151(3_pt_1): p. 758-767.
109. Ware, L.B., et al., Significance of von Willebrand factor in septic and nonseptic patients with acute lung injury. American journal of respiratory and critical care medicine, 2004. 170(7): p. 766-772.
110. Pinsky, D.J., et al., Hypoxia-induced exocytosis of endothelial cell Weibel-Palade bodies. A mechanism for rapid neutrophil recruitment after cardiac preservation. The Journal of clinical investigation, 1996. 97(2): p. 493-500.
111. Mojiri, A., et al., Endothelial cells of different organs exhibit heterogeneity in von Willebrand factor expression in response to hypoxia. Atherosclerosis, 2019. 282: p. 1-10.
112. Batra, K., et al., Diagnosis and interventions of vascular complications in lung transplant. Cardiovascular Diagnosis and Therapy, 2018. 8(3): p. 378.
113. DeFreitas, M.R., et al., Complications of lung transplantation: update on imaging manifestations and management. Radiology: Cardiothoracic Imaging, 2021. 3(4): p. e190252.
114. Inokawa, H., et al., Effects of postmortem heparinization in canine lung transplantation with non–heart-beating donors. The Journal of Thoracic and Cardiovascular Surgery, 2005. 129(2): p. 429-434.
115. Ashorobi, D., M.A. Ameer, and R. Fernandez, Thrombosis. 2019.
116. Wilkerson, W.R. and D.C. Sane. Aging and thrombosis. in Seminars in thrombosis and hemostasis. 2002. Copyright© 2002 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New ….
117. Paramo, J.A., Microvascular thrombosis and clinical implications. Medicina Clínica (English Edition), 2021. 156(12): p. 609-614.
118. Bray, M.A., et al., Microvascular thrombosis: experimental and clinical implications. Translational Research, 2020. 225: p. 105-130.
119. Cushman, M. Epidemiology and risk factors for venous thrombosis. in Seminars in hematology. 2007. Elsevier.
120. Members, W.G., et al., Heart disease and stroke statistics—2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation, 2009. 119(3): p. e21-e181.
121. Yazdanyar, A. and A.B. Newman, The burden of cardiovascular disease in the elderly: morbidity, mortality, and costs. Clinics in geriatric medicine, 2009. 25(4): p. 563-577.
122. Kelly-Hayes, M., Influence of age and health behaviors on stroke risk: lessons from longitudinal studies. Journal of the American Geriatrics Society, 2010. 58: p. S325-S328.
123. Silverstein, M.D., et al., Trends in the incidence of deep vein thrombosis and pulmonary embolism: a 25-year population-based study. Archives of internal medicine, 1998. 158(6): p. 585-593.
124. Boehme, A.K., C. Esenwa, and M.S. Elkind, Stroke risk factors, genetics, and prevention. Circulation research, 2017. 120(3): p. 472-495.
125. Thijssen, D.H., S.E. Carter, and D.J. Green, Arterial structure and function in vascular ageing: are you as old as your arteries? The Journal of physiology, 2016. 594(8): p. 2275-2284.
126. Najjar, S.S., A. Scuteri, and E.G. Lakatta, Arterial aging: is it an immutable cardiovascular risk factor? Hypertension, 2005. 46(3): p. 454-462.

127. Hemmeryckx, B., et al., Age-associated pro-inflammatory adaptations of the mouse thoracic aorta. Thrombosis and haemostasis, 2013. 110(10): p. 785-794.
128. Fleenor, B.S., et al., Superoxide signaling in perivascular adipose tissue promotes age-related artery stiffness. Aging cell, 2014. 13(3): p. 576-578.
129. Morrisette-Thomas, V., et al., Inflamm-aging does not simply reflect increases in pro-inflammatory markers. Mechanisms of ageing and development, 2014. 139: p. 49-57.
130. Xia, S., et al., An update on inflamm-aging: mechanisms, prevention, and treatment. Journal of immunology research, 2016. 2016.
131. Schlaudecker, J. and R. Becker. Inflammatory response and thrombosis in older individuals. in Seminars in thrombosis and hemostasis. 2014. Thieme Medical Publishers.
132. Gleerup, G. and K. Winther, The effect of ageing on platelet function and fibrinolytic activity. Angiology, 1995. 46(8): p. 715-718.
133. Mari, D., et al., Hypercoagulability in centenarians: the paradox of successful aging. 1995.
134. Sepúlveda, C., I. Palomo, and E. Fuentes, Primary and secondary haemostasis changes related to aging. Mechanisms of ageing and development, 2015. 150: p. 46-54.
135. Arya, M., et al., Ultralarge multimers of von Willebrand factor form spontaneous high-strength bonds with the platelet glycoprotein Ib-IX complex: studies using optical tweezers. Blood, The Journal of the American Society of Hematology, 2002. 99(11): p. 3971-3977.
136. Konkle, B.A. Von Willebrand factor and aging. in Seminars in thrombosis and hemostasis. 2014. Thieme Medical Publishers.
137. Atiq, F., et al., Comorbidities associated with higher von Willebrand factor (VWF) levels may explain the age-related increase of VWF in von Willebrand disease. British journal of haematology, 2018. 182(1): p. 93-105.
138. Sadler, J.E., von Willebrand factor. Journal of Biological Chemistry, 1991. 266(34): p. 22777-22780.
139. Collins, C.J., et al., Molecular cloning of the human gene for von Willebrand factor and identification of the transcription initiation site. Proceedings of the National Academy of Sciences, 1987. 84(13): p. 4393-4397.
140. Mayadas, T.N. and D.D. Wagner, von Willebrand Factor Biosynthesis and Processing a. Annals of the New York Academy of Sciences, 1991. 614(1): p. 153-166.
141. Xiang, Y. and J. Hwa, Regulation of VWF expression, and secretion in health and disease. Current opinion in hematology, 2016. 23(3): p. 288-293.
142. Brehm, M.A., Von Willebrand factor processing. Hämostaseologie, 2017. 37(01): p. 59-72.
143. Ruggeri, Z.M. and G.L. Mendolicchio, Adhesion mechanisms in platelet function. Circulation research, 2007. 100(12): p. 1673-1685.
144. Aird, W.C., Endothelium in health and disease. Pharmacological reports, 2008. 60(1): p. 139.
145. Gimbrone Jr, M.A. and G. García-Cardeña, Endothelial cell dysfunction and the pathobiology of atherosclerosis. Circulation research, 2016. 118(4): p. 620-636.
146. Mochizuki, S., et al., Effect of ADAM28 on carcinoma cell metastasis by cleavage of von Willebrand factor. Journal of the National Cancer Institute, 2012. 104(12): p. 906-922.
147. Chauhan, A., et al., Formation of platelet strings and microthrombi in the presence of ADAMTS-13 inhibitor does not require P-selectin or β3 integrin. Journal of thrombosis and haemostasis, 2007. 5(3): p. 583-589.

148. Randi, A. and M. Laffan, Von Willebrand factor and angiogenesis: basic and applied issues. Journal of Thrombosis and Haemostasis, 2017. 15(1): p. 13-20.
149. Randi, A.M., K.E. Smith, and G. Castaman, von Willebrand factor regulation of blood vessel formation. Blood, The Journal of the American Society of Hematology, 2018. 132(2): p. 132-140.
150. Kawecki, C., P. Lenting, and C. Denis, von Willebrand factor and inflammation. Journal of Thrombosis and Haemostasis, 2017. 15(7): p. 1285-1294.
151. Bladbjerg, E., et al., Genetic influence on thrombotic risk markers in the elderly–a Danish twin study. Journal of Thrombosis and Haemostasis, 2006. 4(3): p. 599-607.
152. Murray, G.P., S.R. Post, and G.R. Post, ABO blood group is a determinant of von Willebrand factor protein levels in human pulmonary endothelial cells. Journal of Clinical Pathology, 2020. 73(6): p. 347-349.
153. Higasa, S., T. Tokugawa, and A. Sawada, Diagnosis and management of von Willebrand disease. [Rinsho ketsueki] The Japanese journal of clinical hematology, 2018. 59(10): p. 2222-2232.
154. James, P.D. and A.C. Goodeve, von Willebrand disease. Genetics in Medicine, 2011. 13(5): p. 365-376.
155. Sadler, J., et al., Impact, diagnosis and treatment of von Willebrand disease. Thrombosis and haemostasis, 2000. 84(08): p. 160-174.
156. Othman, M. Platelet-type von Willebrand disease: a rare, often misdiagnosed and underdiagnosed bleeding disorder. in Seminars in thrombosis and hemostasis. 2011. © Thieme Medical Publishers.
157. Yadegari, H. and J. Oldenburg, The current understanding of molecular pathogenesis of quantitative von willebrand disease, types 1 and 3. Hämostaseologie, 2020. 40(01): p. 105-118.
158. Shahidi, M., Thrombosis and von Willebrand factor. Thrombosis and Embolism: from Research to Clinical Practice: Volume 1, 2017: p. 285-306.
159. Wendelboe, A.M. and G.E. Raskob, Global burden of thrombosis: epidemiologic aspects. Circulation research, 2016. 118(9): p. 1340-1347.
160. Barbour, T., et al., Thrombotic microangiopathy and associated renal disorders. Nephrology Dialysis Transplantation, 2012. 27(7): p. 2673-2685.
161. Sadler, J.E., Pathophysiology of thrombotic thrombocytopenic purpura. Blood, The Journal of the American Society of Hematology, 2017. 130(10): p. 1181-1188.
162. Furlan, M., et al., Acquired deficiency of von Willebrand factor-cleaving protease in a patient with thrombotic thrombocytopenic purpura. Blood, The Journal of the American Society of Hematology, 1998. 91(8): p. 2839-2846.
163. Yavuz, B., et al., Potential biomarkers for vascular damage in Alzheimer's disease: Thrombomodulin and von Willebrand factor. The journal of nutrition, health & aging, 2010. 14(6): p. 439-441.
164. Mari, D., et al., Hemostasis abnormalities in patients with vascular dementia and Alzheimer's disease. Thrombosis and haemostasis, 1996. 75(02): p. 216-218.
165. De Oliveira, C.O., et al., Plasma von Willebrand factor levels correlate with clinical outcome of severe traumatic brain injury. Journal of neurotrauma, 2007. 24(8): p. 1331-1338.

166. El Basset Abo El Ezz, A.A., et al., The predictive value of Von Willebrand factor antigen plasma levels in children with acute lung injury. Pediatric pulmonology, 2017. 52(1): p. 91-97.
167. van der Vorm, L.N., et al., Circulating active von Willebrand factor levels are increased in chronic kidney disease and end-stage renal disease. Clinical kidney journal, 2020. 13(1): p. 72.
168. Ito, Y., et al., Age-related changes in the hepatic microcirculation in mice. Experimental Gerontology, 2007. 42(8): p. 789-797.
169. Hilmer, S.N., et al., Age-related changes in the hepatic sinusoidal endothelium impede lipoprotein transfer in the rat. Hepatology, 2005. 42(6): p. 1349-1354.
170. Cogger, V.C., et al., Hepatic sinusoidal pseudocapillarization with aging in the non-human primate. Experimental gerontology, 2003. 38(10): p. 1101-1107.
171. Coppola, R., et al., Von Willebrand factor in Italian centenarians. 2003.
172. Albánez, S., et al., Aging and ABO blood type influence von Willebrand factor and factor VIII levels through interrelated mechanisms. Journal of Thrombosis and Haemostasis, 2016. 14(5): p. 953-963.
173. Biguzzi, E., et al., Rise of levels of von Willebrand factor and factor VIII with age: role of genetic and acquired risk factors. Thrombosis Research, 2021. 197: p. 172-178.
174. Miesbach, W., et al., Age-dependent increase of FVIII: C in mild haemophilia A. Haemophilia, 2009. 15(5): p. 1022-1026.
175. Rejtő, J., et al., Influence of blood group, von Willebrand factor levels, and age on factor VIII levels in non-severe haemophilia A. Journal of Thrombosis and Haemostasis, 2020. 18(5): p. 1081-1086.
176. Kokame, K., et al., von Willebrand factor-to-ADAMTS13 ratio increases with age in a Japanese population. Journal of Thrombosis and Haemostasis, 2011. 9(7): p. 1426-1428.
177. Al-Awadhi, A.M., et al., Investigating the influence of age, gender and ABO blood group on ADAMTS-13 antigen and activity levels in healthy Arabs. Blood Transfusion, 2014. 12(1): p. 138.
178. Davies, J.A., et al., von Willebrand factor: demographics of plasma protein level in a large blood donor cohort from South Wales in the United Kingdom. Haemophilia: the Official Journal of the World Federation of Hemophilia, 2012. 18(3): p. e79-81.
179. Kadir, R.A., et al., Variations in coagulation factors in women: effects of age, ethnicity, menstrual cycle and combined oral contraceptive. Thrombosis and haemostasis, 1999. 82(11): p. 1456-1461.
180. Blombäck, M., et al., Preanalytical conditions that affect coagulation testing, including hormonal status and therapy. Journal of Thrombosis and Haemostasis, 2007. 5(4): p. 855-858.
181. Chapin, J., Von Willebrand disease in the elderly: clinical perspectives. Clinical interventions in aging, 2018: p. 1531-1541.
182. KAVOUSI, M., et al., von Willebrand factor plasma levels, genetic variations and coronary heart disease in an older population. Journal of Thrombosis and Haemostasis, 2012. 10(7): p. 1262-1269.
183. Habe, K., et al., Plasma ADAMTS13, von Willebrand factor (VWF) and VWF propeptide profiles in patients with DIC and related diseases. Thrombosis research, 2012. 129(5): p. 598-602.

184. Conroy, A.L., et al., Endothelium-based biomarkers are associated with cerebral malaria in Malawian children: a retrospective case-control study. PloS one, 2010. 5(12): p. e15291.
185. Müller, A.M., et al., Correlation of age with in vivo expression of endothelial markers. Experimental gerontology, 2002. 37(5): p. 713-719.
186. Mohebali, D., et al., Alterations in platelet function during aging: clinical correlations with thromboinflammatory disease in older adults. Journal of the American Geriatrics Society, 2014. 62(3): p. 529-535.
187. Zhou, X.J., et al., Association of renal injury with nitric oxide deficiency in aged SHR: prevention by hypertension control with AT1 blockade. Kidney international, 2002. 62(3): p. 914-921.
188. Pie, J.-E., et al., Age-related decline of inducible nitric oxide synthase gene expression in primary cultured rat hepatocytes. Molecules and cells, 2002. 13(3): p. 399-406.
189. Torregrossa, A.C., M. Aranke, and N.S. Bryan, Nitric oxide and geriatrics: Implications in diagnostics and treatment of the elderly. Journal of geriatric cardiology: JGC, 2011. 8(4): p. 230.
190. O'Donnell, J.S., Low VWF: insights into pathogenesis, diagnosis, and clinical management. Blood Advances, 2020. 4(13): p. 3191-3199.
191. Seaman, C.D. and M.V. Ragni, The association of aging with von Willebrand factor levels and bleeding risk in type 1 von Willebrand disease. Clinical and Applied Thrombosis/Hemostasis, 2018. 24(3): p. 434-438.
192. Seaman, C.D. and M.V. Ragni, The effect of age on von Willebrand factor and bleeding symptoms in von Willebrand disease. Thrombosis and Haemostasis, 2020. 120(08): p. 1159-1165.
193. Abou-Ismail, M.Y., et al., Outgrowing the laboratory diagnosis of type 1 von W illebrand disease: A two decade study. American Journal of Hematology, 2018. 93(2): p. 232-237.
194. Rydz, N., et al., Changes in von Willebrand factor level and von Willebrand activity with age in type 1 von Willebrand disease. Haemophilia, 2015. 21(5): p. 636-641.
195. Sanders, Y.V., et al., von Willebrand disease and aging: an evolving phenotype. Journal of Thrombosis and Haemostasis, 2014. 12(7): p. 1066-1075.
196. Birch, K.A., et al., Calcium/calmodulin transduces thrombin-stimulated secretion: studies in intact and minimally permeabilized human umbilical vein endothelial cells. The Journal of cell biology, 1992. 118(6): p. 1501-1510.
197. Bhatia, R., et al., Ceramide triggers Weibel–Palade body exocytosis. Circulation research, 2004. 95(3): p. 319-324.
198. Matsushita, K., C.N. Morrell, and C.J. Lowenstein, Sphingosine 1-phosphate activates Weibel-Palade body exocytosis. Proceedings of the National Academy of Sciences, 2004. 101(31): p. 11483-11487.
199. Matsushita, K., et al., Vascular endothelial growth factor regulation of Weibel-Palade–body exocytosis. Blood, 2005. 105(1): p. 207-214.
200. Vischer, U.M. and P. de Moerloose, von Willebrand factor: from cell biology to the clinical management of von Willebrand's disease. Critical reviews in oncology/hematology, 1999. 30(2): p. 93-109.
201. Chung, H.Y., et al., Redefining chronic inflammation in aging and age-related diseases: proposal of the senoinflammation concept. Aging and disease, 2019. 10(2): p. 367.
202. Sanada, F., et al., Source of chronic inflammation in aging. Frontiers in cardiovascular medicine, 2018. 5: p. 12.

203. Branchford, B.R. and S.L. Carpenter, The role of inflammation in venous thromboembolism. Frontiers in pediatrics, 2018. 6: p. 142.
204. Ferrucci, L. and E. Fabbri, Inflammageing: chronic inflammation in ageing, cardiovascular disease, and frailty. Nature Reviews Cardiology, 2018. 15(9): p. 505-522.
205. Newman, A.B., et al., Trajectories of function and biomarkers with age: the CHS All Stars Study. International journal of epidemiology, 2016. 45(4): p. 1135-1145.
206. Donato, A.J., et al., Vascular endothelial dysfunction with aging: endothelin-1 and endothelial nitric oxide synthase. American Journal of Physiology-Heart and Circulatory Physiology, 2009. 297(1): p. H425-H432.
207. Matsushita, K., et al., Nitric oxide regulates exocytosis by S-nitrosylation of N-ethylmaleimide-sensitive factor. Cell, 2003. 115(2): p. 139-150.
208. Nakayama, T., et al., Endothelial von Willebrand factor release due to eNOS deficiency predisposes to thrombotic microangiopathy in mouse aging kidney. The American journal of pathology, 2010. 176(5): p. 2198-2208.
209. Afanas'ev, I., Superoxide and nitric oxide in senescence and aging. Front Biosci, 2009. 14: p. 3899-3912.
210. Leitner, G.C., et al., Effects of endothelin-1 and phenylephrine on plasma levels of von Willebrand factor and protein S. Thrombosis Research, 2010. 125(1): p. e5-e8.
211. Somanath, P.R., et al., Deficiency in core circadian protein Bmal1 is associated with a prothrombotic and vascular phenotype. Journal of cellular physiology, 2011. 226(1): p. 132-140.
212. Hemmeryckx, B., et al., Progression of the prothrombotic state in aging Bmal1-deficient mice. Arteriosclerosis, thrombosis, and vascular biology, 2011. 31(11): p. 2552-2559.
213. Duncan, M.J., et al., Influence of aging on Bmal1 and Per2 expression in extra-SCN oscillators in hamster brain. Brain research, 2013. 1491: p. 44-53.
214. McKinley, M.J., et al., Differential effects of aging on fluid intake in response to hypovolemia, hypertonicity, and hormonal stimuli in Munich Wistar rats. Proceedings of the National Academy of Sciences, 2006. 103(9): p. 3450-3455.
215. Begg, D.P., Disturbances of thirst and fluid balance associated with aging. Physiology & behavior, 2017. 178: p. 28-34.
216. Miescher, E. and S.M. Fortney, Responses to dehydration and rehydration during heat exposure in young and older men. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 1989. 257(5): p. R1050-R1056.
217. Coppola, R., et al., Von Willebrand factor in Italian centenarians. Haematologica, 2003. 88(1): p. 39-43.
218. Tsai, H.-M., I.I. Sussman, and R.L. Nagel, Shear stress enhances the proteolysis of von Willebrand factor in normal plasma. 1994.
219. Carallo, C., et al., Carotid endothelial shear stress reduction with aging is associated with plaque development in twelve years. Atherosclerosis, 2016. 251: p. 63-69.
220. van den Dries, L.W., et al., von Willebrand Factor is elevated in HIV patients with a history of thrombosis. Frontiers in microbiology, 2015. 6: p. 180.
221. O'regan, N., et al., A novel role for von Willebrand factor in the pathogenesis of experimental cerebral malaria. Blood, The Journal of the American Society of Hematology, 2016. 127(9): p. 1192-1201.

222. Chen, H., et al., Elevated levels of von Willebrand factor and high mobility group box 1 (HMGB1) are associated with disease severity and clinical outcome of scrub typhus. International Journal of Infectious Diseases, 2017. 61: p. 114-120.
223. Djamiatun, K., et al., Severe dengue is associated with consumption of von Willebrand factor and its cleaving enzyme ADAMTS-13. PLoS neglected tropical diseases, 2012. 6(5): p. e1628.
224. Shi, Y., et al., COVID-19 infection: the perspectives on immune responses. 2020, Nature Publishing Group.
225. Robba, C., et al., Multiple organ dysfunction in SARS-CoV-2: MODS-CoV-2. Expert review of respiratory medicine, 2020. 14(9): p. 865-868.
226. Yang, L., et al., COVID-19: immunopathogenesis and Immunotherapeutics. Signal transduction and targeted therapy, 2020. 5(1): p. 1-8.
227. Tang, N., et al., Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia. Journal of thrombosis and haemostasis, 2020. 18(4): p. 844-847.
228. Bazzan, M., et al., Low ADAMTS 13 plasma levels are predictors of mortality in COVID-19 patients. Internal and Emergency Medicine, 2020. 15(5): p. 861-863.
229. Mei, Z.W., et al., Role of von Willebrand Factor in COVID-19 Associated Coagulopathy. The journal of applied laboratory medicine, 2021.
230. Helms, J., et al., High risk of thrombosis in patients with severe SARS-CoV-2 infection: a multicenter prospective cohort study. Intensive care medicine, 2020. 46(6): p. 1089-1098.
231. Klok, F., et al., Incidence of thrombotic complications in critically ill ICU patients with COVID-19. Thrombosis research, 2020. 191: p. 145-147.
232. Klok, F., et al., Confirmation of the high cumulative incidence of thrombotic complications in critically ill ICU patients with COVID-19: an updated analysis. Thrombosis research, 2020. 191: p. 148-150.
233. Middeldorp, S., et al., Incidence of venous thromboembolism in hospitalized patients with COVID-19. Journal of Thrombosis and Haemostasis, 2020. 18(8): p. 1995-2002.
234. Poissy, J., et al., Pulmonary embolism in patients with COVID-19: awareness of an increased prevalence. Circulation, 2020. 142(2): p. 184-186.
235. Bonaventura, A., et al., Endothelial dysfunction and immunothrombosis as key pathogenic mechanisms in COVID-19. Nature Reviews Immunology, 2021. 21(5): p. 319-329.
236. Liu, P.P., et al., The science underlying COVID-19: implications for the cardiovascular system. Circulation, 2020. 142(1): p. 68-78.
237. Becker, R.C., et al., COVID-19 and biomarkers of thrombosis: focus on von Willebrand factor and extracellular vesicles. Journal of Thrombosis and Thrombolysis, 2021. 52: p. 1010-1019.
238. Chandel, A., et al., Association of D-dimer and Fibrinogen With Hypercoagulability in COVID-19 Requiring Extracorporeal Membrane Oxygenation. Journal of intensive care medicine, 2021. 36(6): p. 689-695.
239. Küçükceran, K., et al., Predictive value of D-dimer/albumin ratio and fibrinogen/albumin ratio for in-hospital mortality in patients with COVID-19. International journal of clinical practice, 2021: p. e14263.
240. Goshua, G., et al., Endotheliopathy in COVID-19-associated coagulopathy: evidence from a single-centre, cross-sectional study. The Lancet Haematology, 2020. 7(8): p. e575-e582.

241. Bashir, D.A., et al., Secretion of von Willebrand Factor and Suppression of ADAMTS-13 Activity by Markedly High Concentration of Ferritin. Clinical and Applied Thrombosis/Hemostasis, 2021. 27: p. 1076029621992128.
242. Ward, S.E., et al., ADAMTS13 regulation of VWF multimer distribution in severe COVID-19. Journal of Thrombosis and Haemostasis, 2021.
243. Patel, B.V., et al., Pulmonary angiopathy in severe COVID-19: physiologic, imaging, and hematologic observations. American journal of respiratory and critical care medicine, 2020. 202(5): p. 690-699.
244. Iba, T., et al., Coagulopathy of coronavirus disease 2019. Critical care medicine, 2020.
245. Tiwari, N.R., et al., COVID-19 and thrombotic microangiopathies. Thrombosis research, 2021.
246. Mancini, I., et al., The ADAMTS13-von Willebrand factor axis in COVID-19 patients. Journal of Thrombosis and Haemostasis, 2021. 19(2): p. 513-521.
247. Doevelaar, A.A., et al., von Willebrand factor multimer formation contributes to immunothrombosis in coronavirus disease 2019. Critical Care Medicine, 2021. 49(5): p. e512-e520.
248. Panigada, M., et al., Hypercoagulability of COVID-19 patients in intensive care unit: a report of thromboelastography findings and other parameters of hemostasis. Journal of Thrombosis and Haemostasis, 2020. 18(7): p. 1738-1742.
249. Henry, B.M., et al., ADAMTS13 activity to von Willebrand factor antigen ratio predicts acute kidney injury in patients with COVID-19: Evidence of SARS-CoV-2 induced secondary thrombotic microangiopathy. International journal of laboratory hematology, 2020.
250. Morici, N., et al., Role of von Willebrand factor and ADAMTS-13 in the pathogenesis of thrombi in SARS-CoV-2 infection: time to rethink. Thrombosis and haemostasis, 2020. 120(09): p. 1339-1342.
251. Nicolai, L., et al., Immunothrombotic dysregulation in COVID-19 pneumonia is associated with respiratory failure and coagulopathy. Circulation, 2020. 142(12): p. 1176-1189.
252. Yang, J., et al., Insights into immunothrombosis: The interplay among Neutrophil Extracellular Trap, von Willebrand factor, and ADAMTS13. Frontiers in Immunology, 2020. 11.
253. Aksenova, A.Y., Von Willebrand factor and endothelial damage: a possible association with COVID-19. Ecological genetics, 2020. 18(2): p. 135-138.
254. Gheblawi, M., et al., Angiotensin-converting enzyme 2: SARS-CoV-2 receptor and regulator of the renin-angiotensin system: celebrating the 20th anniversary of the discovery of ACE2. Circulation research, 2020. 126(10): p. 1456-1474.
255. Hamming, I., et al., Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. The Journal of Pathology: A Journal of the Pathological Society of Great Britain and Ireland, 2004. 203(2): p. 631-637.
256. Letko, M., A. Marzi, and V. Munster, Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. Nature microbiology, 2020. 5(4): p. 562-569.
257. Amraei, R., et al., CD209L/L-SIGN and CD209/DC-SIGN act as receptors for SARS-CoV-2. ACS Central Science, 2021. 7(7): p. 1156-1165.

258. Fraga-Silva, R.A., et al., The angiotensin-converting enzyme 2/angiotensin-(1–7)/Mas receptor axis: a potential target for treating thrombotic diseases. Thrombosis and haemostasis, 2012. 108(12): p. 1089-1096.
259. Monteil, V., et al., Inhibition of SARS-CoV-2 infections in engineered human tissues using clinical-grade soluble human ACE2. Cell, 2020. 181(4): p. 905-913. e7.
260. Samavati, L. and B.D. Uhal, ACE2, much more than just a receptor for SARS-COV-2. Frontiers in cellular and infection microbiology, 2020. 10: p. 317.
261. Cugno, M., et al., Complement activation and endothelial perturbation parallel COVID-19 severity and activity. Journal of autoimmunity, 2021. 116: p. 102560.
262. Roberts, K.A., et al., Vascular manifestations of COVID-19–thromboembolism and microvascular dysfunction. Frontiers in cardiovascular medicine, 2020. 7: p. 215.
263. Fletcher-Sandersjöö, A. and B.-M. Bellander, Is COVID-19 associated thrombosis caused by overactivation of the complement cascade? A literature review. Thrombosis research, 2020. 194: p. 36-41.
264. Yan, G., et al., Tumor necrosis factor-α downregulates endothelial nitric oxide synthase mRNA stability via translation elongation factor 1-α 1. Circulation research, 2008. 103(6): p. 591-597.
265. Xiao, J., et al., EPAC regulates von Willebrand factor secretion from endothelial cells in a PI3K/eNOS-dependent manner during inflammation. bioRxiv, 2020.
266. Fang, W., et al., The role of NO in COVID-19 and potential therapeutic strategies. Free Radical Biology and Medicine, 2021. 163: p. 153-162.
267. Ozdemir, B. and A. Yazici, Could the decrease in the endothelial nitric oxide (NO) production and NO bioavailability be the crucial cause of COVID-19 related deaths? Medical Hypotheses, 2020. 144: p. 109970.
268. Huisman, A., et al., Involvement of ADAMTS13 and von Willebrand factor in thromboembolic events in patients infected with SARS-CoV-2. International Journal of Laboratory Hematology, 2020. 42(5): p. e211.
269. Rauch, A., et al., Coagulation biomarkers are independent predictors of increased oxygen requirements in COVID-19. Journal of Thrombosis and Haemostasis, 2020. 18(11): p. 2942-2953.
270. Ladikou, E.E., et al., Von Willebrand factor (vWF): marker of endothelial damage and thrombotic risk in COVID-19? Clinical Medicine, 2020. 20(5): p. e178.
271. Ward, S.E., et al., Von Willebrand factor propeptide in severe coronavirus disease 2019 (COVID-19): evidence of acute and sustained endothelial cell activation. British journal of haematology, 2021. 192(4): p. 714-719.
272. Philippe, A., et al., Circulating Von Willebrand factor and high molecular weight multimers as markers of endothelial injury predict COVID-19 in-hospital mortality. Angiogenesis, 2021: p. 1-13.
273. Delrue, M., et al., von Willebrand factor/ADAMTS13 axis and venous thromboembolism in moderate-to-severe COVID-19 patients. British journal of haematology, 2021. 192(6): p. 1097-1100.
274. Taus, F., et al., Platelets promote thromboinflammation in SARS-CoV-2 pneumonia. Arteriosclerosis, Thrombosis, and Vascular Biology, 2020. 40(12): p. 2975-2989.
275. Marco, A. and P. Marco, Von Willebrand factor and ADAMTS13 activity as clinical severity markers in patients with COVID-19. Journal of thrombosis and thrombolysis, 2021. 52(2): p. 497-503.

276. De Cristofaro, R., et al., Marked von Willebrand factor and factor VIII elevations in severe acute respiratory syndrome coronavirus-2-positive, but not severe acute respiratory syndrome coronavirus-2-negative, pneumonia: a case–control study. Blood Coagulation & Fibrinolysis, 2021. 32(4): p. 285-289.
277. Blasi, A., et al., In vitro hypercoagulability and ongoing in vivo activation of coagulation and fibrinolysis in COVID-19 patients on anticoagulation. Journal of Thrombosis and Haemostasis, 2020. 18(10): p. 2646-2653.
278. Ruberto, F., et al., Von Willebrand factor with increased binding capacity is associated with reduced platelet aggregation but enhanced agglutination in COVID-19 patients: another COVID-19 paradox? Journal of Thrombosis and Thrombolysis, 2021: p. 1-6.
279. Sweeney, J.M., et al., Evidence for secondary thrombotic microangiopathy in COVID-19. MedRxiv, 2020.
280. Rodríguez, M.R., et al., Increased von Willebrand factor antigen and low ADAMTS13 activity are related to poor prognosis in covid-19 patients. International Journal of Laboratory Hematology, 2021. 43(4): p. O152.
281. Hardy, M., et al., Prothrombotic hemostasis disturbances in patients with severe COVID-19: Individual daily data. Data in brief, 2020. 33: p. 106519.
282. Masi, P., et al., Systemic inflammatory response syndrome is a major contributor to COVID-19–associated coagulopathy: insights from a prospective, single-center cohort study. Circulation, 2020. 142(6): p. 611-614.
283. von Meijenfeldt, F.A., et al., Prothrombotic changes in patients with COVID-19 are associated with disease severity and mortality. Research and practice in thrombosis and haemostasis, 2021. 5(1): p. 132-141.
284. De Jongh, R., et al., Vascular activation is a strong predictor of mortality in coronavirus disease 2019 patients on the ICU. Blood Coagulation & Fibrinolysis, 2021. 32(4): p. 290-293.
285. Pascreau, T., et al., ADAMTS 13 deficiency is associated with abnormal distribution of von Willebrand factor multimers in patients with COVID-19. Thrombosis Research, 2021. 204: p. 138.
286. Liu, N., et al., The impact of ABO blood group on COVID-19 infection risk and mortality: A systematic review and meta-analysis. Blood Reviews, 2021. 48: p. 100785.
287. Muñiz-Diaz, E., et al., Relationship between the ABO blood group and COVID-19 susceptibility, severity and mortality in two cohorts of patients. Blood Transfusion, 2021. 19(1): p. 54.
288. Kageyama, S., et al., Pharmacokinetics and pharmacodynamics of AJW200, a humanized monoclonal antibody to von Willebrand factor, in monkeys. Arteriosclerosis, thrombosis, and vascular biology, 2002. 22(1): p. 187-192.
289. De Meyer, S.F., et al., Von Willebrand factor: drug and drug target. Cardiovascular & Haematological Disorders-Drug Targets (Formerly Current Drug Targets-Cardiovascular & Hematological Disorders), 2009. 9(1): p. 9-20.
290. De Meyer, S.F., et al., von Willebrand factor: an emerging target in stroke therapy. Stroke, 2012. 43(2): p. 599-606.
291. Scully, M., et al., Caplacizumab treatment for acquired thrombotic thrombocytopenic purpura. New England Journal of Medicine, 2019. 380(4): p. 335-346.

292. Knoebl, P., et al., Efficacy and safety of open-label caplacizumab in patients with exacerbations of acquired thrombotic thrombocytopenic purpura in the HERCULES study. Journal of thrombosis and haemostasis, 2020. 18(2): p. 479-484.
293. Xu, X.R., et al., Platelets and platelet adhesion molecules: novel mechanisms of thrombosis and anti-thrombotic therapies. Thrombosis journal, 2016. 14(1): p. 37-46.
294. Zheng, L., et al., Therapeutic efficacy of the platelet glycoprotein Ib antagonist anfibatide in murine models of thrombotic thrombocytopenic purpura. Blood advances, 2016. 1(1): p. 75-83.
295. Plaimauer, B., et al., Recombinant ADAMTS13 normalizes von Willebrand factor-cleaving activity in plasma of acquired TTP patients by overriding inhibitory antibodies. journal of Thrombosis and Haemostasis, 2011. 9(5): p. 936-944.
296. Chen, J., et al., N-acetylcysteine reduces the size and activity of von Willebrand factor in human plasma and mice. The Journal of clinical investigation, 2011. 121(2): p. 593-603.
297. Rottenstreich, A., et al., The role of N-acetylcysteine in the treatment of thrombotic thrombocytopenic purpura. Journal of thrombosis and thrombolysis, 2016. 41(4): p. 678-683.
298. Sakai, K., et al., Novel aptamer to von Willebrand factor A1 domain (TAGX-0004) shows total inhibition of thrombus formation superior to ARC1779 and comparable to caplacizumab. haematologica, 2020. 105(11).
299. Kovacevic, K.D., et al., The aptamer BT200 effectively inhibits von Willebrand factor (VWF) dependent platelet function after stimulated VWF release by desmopressin or endotoxin. Scientific reports, 2020. 10(1): p. 1-9.
300. Jackson, C.F. and N.K. Wenger, Cardiovascular disease in the elderly. Revista Española de Cardiología (English Edition), 2011. 64(8): p. 697-712.
301. Xu, X., et al., Age-related impairment of vascular structure and functions. Aging and disease, 2017. 8(5): p. 590.
302. Parkes, I., S. Chintawar, and M.Z. Cader, Neurovascular dysfunction in dementia–human cellular models and molecular mechanisms. Clinical Science, 2018. 132(3): p. 399-418.
303. Román, G.C., et al., Vascular cognitive disorder: a new diagnostic category updating vascular cognitive impairment and vascular dementia. Journal of the neurological sciences, 2004. 226(1-2): p. 81-87.
304. Verhamme, P. and M. Hoylaerts, The pivotal role of the endothelium in haemostasis and thrombosis. Acta Clinica Belgica, 2006. 61(5): p. 213-219.
305. Denis, C.V., Molecular and cellular biology of von Willebrand factor. International journal of hematology, 2002. 75: p. 3-8.
306. O'Sullivan, J.M., et al. Emerging roles for von Willebrand factor in cancer cell biology. in Seminars in thrombosis and hemostasis. 2018. Thieme Medical Publishers.
307. Denis, C., et al., A mouse model of severe von Willebrand disease: defects in hemostasis and thrombosis. Proceedings of the National Academy of Sciences, 1998. 95(16): p. 9524-9529.
308. Springer, T.A., von Willebrand factor, Jedi knight of the bloodstream. Blood, The Journal of the American Society of Hematology, 2014. 124(9): p. 1412-1425.
309. Chen, J. and D.W. Chung, Inflammation, von Willebrand factor, and ADAMTS13. Blood, 2018. 132(2): p. 141-147.
310. Luo, G.-P., et al., von Willebrand factor: more than a regulator of hemostasis and thrombosis. Acta haematologica, 2012. 128(3): p. 158-169.

311. Mojiri, A., P. Alavi, and N. Jahroudi, Von Willebrand factor contribution to pathophysiology outside of von Willebrand disease. Microcirculation, 2019. 26(4): p. e12510.
312. Terraube, V., et al., Increased metastatic potential of tumor cells in von Willebrand factor-deficient mice. Journal of Thrombosis and Haemostasis, 2006. 4(3): p. 519-526.
313. Chen, J. and J.A. López, Interactions of platelets with subendothelium and endothelium. Microcirculation, 2005. 12(3): p. 235-246.
314. Stockschlaeder, M., R. Schneppenheim, and U. Budde, Update on von Willebrand factor multimers: focus on high-molecular-weight multimers and their role in hemostasis. Blood Coagulation & Fibrinolysis, 2014. 25(3): p. 206.
315. Falter, T., et al., A novel von Willebrand factor multimer ratio as marker of disease activity in thrombotic thrombocytopenic purpura. Blood Advances, 2023.
316. Denis, C.V., et al., Clearance of von Willebrand factor. Thrombosis and haemostasis, 2008. 99(02): p. 271-278.
317. Dong, J.F., Cleavage of ultra-large von Willebrand factor by ADAMTS-13 under flow conditions. Journal of thrombosis and haemostasis, 2005. 3(8): p. 1710-1716.
318. Lip, G. and A.D. Blann, von Willebrand factor and its relevance to cardiovascular disorders. Heart, 1995. 74(6): p. 580-583.
319. Kalagara, T., et al., The endothelial glycocalyx anchors von Willebrand factor fibers to the vascular endothelium. Blood advances, 2018. 2(18): p. 2347-2357.
320. James, P.D. and D. Lillicrap, von Willebrand disease: clinical and laboratory lessons learned from the large von Willebrand disease studies. American journal of hematology, 2012. 87(S1): p. S4-S11.
321. Van Schie, M., et al., Genetic determinants of von Willebrand factor levels and activity in relation to the risk of cardiovascular disease: a review. Journal of Thrombosis and Haemostasis, 2011. 9(5): p. 899-908.
322. Wiman, B.r., et al., Plasma levels of tissue plasminogen activator/plasminogen activator inhibitor-1 complex and von Willebrand factor are significant risk markers for recurrent myocardial infarction in the Stockholm Heart Epidemiology Program (SHEEP) study. Arteriosclerosis, thrombosis, and vascular biology, 2000. 20(8): p. 2019-2023.
323. Borghi, M., et al., Increase of von Willebrand factor with aging in type 1 von Willebrand disease: fact or fiction? Haematologica, 2017. 102(11): p. e431.
324. Royall, D.R., et al., Serum protein mediators of dementia and aging proper. Aging (Albany NY), 2016. 8(12): p. 3241.
325. Xiang, Y. and J. Hwa, Regulation of VWF expression, and secretion in health and disease. Current opinion in hematology, 2016. 23(3): p. 288.
326. Thomazini, C.M., et al., Optimization of von Willebrand factor multimer analysis in vertical mini-gel electrophoresis systems: A rapid procedure. Thrombosis Research, 2019. 175: p. 76-83.
327. Biran, A., Z. Porat, and V. Krizhanovsky, Quantitative Identification of Senescent Cells in Cancer, in Cancer Immunosurveillance. 2019, Springer. p. 259-267.
328. Biran, A., et al., Quantitative identification of senescent cells in aging and disease. Aging cell, 2017. 16(4): p. 661-671.
329. Harrison, R.L. and P.A. McKee, Estrogen stimulates von Willebrand factor production by cultured endothelial cells, 1984. 63(3):657-64.

330. Furlan, M., Von Willebrand factor: molecular size and functional activity. Annals of hematology, 1996. 72: p. 341-348.
331. Aird, W.C., Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. Circulation research, 2007. 100(2): p. 158-173.
332. Aird, W.C., Phenotypic heterogeneity of the endothelium: II. Representative vascular beds. Circulation research, 2007. 100(2): p. 174-190.
333. Pusztaszeri, M.P., W. Seelentag, and F.T. Bosman, Immunohistochemical expression of endothelial markers CD31, CD34, von Willebrand factor, and Fli-1 in normal human tissues. Journal of Histochemistry & Cytochemistry, 2006. 54(4): p. 385-395.
334. Yamamoto, K., et al., Tissue distribution and regulation of murine von Willebrand factor gene expression in vivo. Blood, The Journal of the American Society of Hematology, 1998. 92(8): p. 2791-2801.
335. Van Deursen, J.M., The role of senescent cells in ageing. Nature, 2014. 509(7501): p. 439-446.
336. Matjusaitis, M., et al., Biomarkers to identify and isolate senescent cells. Ageing research reviews, 2016. 29: p. 1-12.
337. O'Brate, A. and P. Giannakakou, The importance of p53 location: nuclear or cytoplasmic zip code? Drug resistance updates, 2003. 6(6): p. 313-322.
338. Spiel, A.O., J.C. Gilbert, and B. Jilma, von Willebrand factor in cardiovascular disease: focus on acute coronary syndromes. Circulation, 2008. 117(11): p. 1449-1459.
339. Palta, S., R. Saroa, and A. Palta, Overview of the coagulation system. Indian journal of anaesthesia, 2014. 58(5): p. 515.
340. Jackson, S.P., The growing complexity of platelet aggregation. Blood, The Journal of the American Society of Hematology, 2007. 109(12): p. 5087-5095.
341. Franchini, M., Hemostasis and aging. Critical reviews in oncology/hematology, 2006. 60(2): p. 144-151.
342. Müller, A.M., et al., Expression of the endothelial markers PECAM-1, vWf, and CD34 in vivo and in vitro. Experimental and molecular pathology, 2002. 72(3): p. 221-229.
343. Yuan, L., et al., A role of stochastic phenotype switching in generating mosaic endothelial cell heterogeneity. Nature communications, 2016. 7(1): p. 1-16.
344. Swieringa, F., et al., Integrating platelet and coagulation activation in fibrin clot formation. Research and practice in thrombosis and haemostasis, 2018. 2(3): p. 450-460.
345. De Silva, T.M. and F.M. Faraci, Microvascular dysfunction and cognitive impairment. Cellular and molecular neurobiology, 2016. 36(2): p. 241-258.
346. Hayon, I.L. and Y. Haupt, p53: an internal investigation. Cell Cycle, 2002. 1(2): p. 105-110.
347. Roger, L., F. Tomas, and V. Gire, Mechanisms and regulation of cellular senescence. International journal of molecular sciences, 2021. 22(23): p. 13173.
348. Mojiri, A., et al., Telomerase therapy reverses vascular senescence and extends lifespan in progeria mice. European Heart Journal, 2021. 42(42): p. 4352-4369.
349. Luo, X., et al., Sirtuin 1 ameliorates defenestration in hepatic sinusoidal endothelial cells during liver fibrosis via inhibiting stress-induced premature senescence. Cell proliferation, 2021. 54(3): p. e12991.
350. Sinha, J.K., S. Ghosh, and M. Raghunath, Progeria: a rare genetic premature ageing disorder. Indian Journal of Medical Research, 2014. 139(5): p. 667-674.

351. McClintock, D., L.B. Gordon, and K. Djabali, Hutchinson–Gilford progeria mutant lamin A primarily targets human vascular cells as detected by an anti-Lamin A G608G antibody. Proceedings of the national academy of sciences, 2006. 103(7): p. 2154-2159.
352. Gordon, L.B., et al., Disease progression in Hutchinson-Gilford progeria syndrome: impact on growth and development. Pediatrics, 2007. 120(4): p. 824-833.
353. Olive, M., et al., Cardiovascular pathology in Hutchinson-Gilford progeria: correlation with the vascular pathology of aging. Arteriosclerosis, thrombosis, and vascular biology, 2010. 30(11): p. 2301-2309.
354. Taimen, P., et al., A progeria mutation reveals functions for lamin A in nuclear assembly, architecture, and chromosome organization. Proceedings of the National Academy of Sciences, 2009. 106(49): p. 20788-20793.
355. Scaffidi, P. and T. Misteli, Lamin A-dependent nuclear defects in human aging. Science, 2006. 312(5776): p. 1059-1063.
356. Ashapkin, V.V., L.I. Kutueva, and I.I. Kireev, Are there common mechanisms between the Hutchinson–Gilford progeria syndrome and natural aging? Frontiers in genetics, 2019. 10: p. 451635.
357. Lamis, A., et al., Hutchinson-Gilford progeria syndrome: a literature review. Cureus, 2022. 14(8).
358. Xu, Q., et al., Vascular senescence in progeria: Role of endothelial dysfunction. European Heart Journal Open, 2022. 2(4): p. oeac047.
359. Peacock, A.J., R. Naeije, and L.J. Rubin, Pulmonary circulation. 2010: Hodder Arnold.
360. Townsley, M.I., Structure and composition of pulmonary arteries, capillaries and veins. Comprehensive Physiology, 2012. 2: p. 675.
361. Kandathil, A. and M. Chamarthy, Pulmonary vascular anatomy & anatomical variants. Cardiovascular diagnosis and therapy, 2018. 8(3): p. 201.
362. Konstantinov, I.E., At the cutting edge of the impossible: a tribute to Vladimir P. Demikhov. Texas Heart Institute Journal, 2009. 36(5): p. 453.
363. Hardy, J.D., et al., Lung homotransplantation in man: report of the initial case. Jama, 1963. 186(12): p. 1065-1074.
364. Field, M.J., R.L. Lawrence, and L. Zwanziger, Immunosuppressive Drugs for Transplant Patients, in Extending Medicare Coverage for Preventive and Other Services. 2000, National Academies Press (US).
365. Richmond, C., Norman Shumway. BMJ: British Medical Journal, 2006. 332(7540): p. 553.
366. Group*, T.L.T., Unilateral lung transplantation for pulmonary fibrosis. New England Journal of Medicine, 1986. 314(18): p. 1140-1145.
367. Patterson, G., et al., Technique of successful clinical double-lung transplantation. The Annals of thoracic surgery, 1988. 45(6): p. 626-633.
368. Corris, P.A. Lung transplantation for cystic fibrosis and bronchiectasis. in Seminars in respiratory and critical care medicine. 2013. Thieme Medical Publishers.
369. Weill, D., et al., A consensus document for the selection of lung transplant candidates: 2014—an update from the Pulmonary Transplantation Council of the International Society for Heart and Lung Transplantation. The Journal of Heart and Lung Transplantation, 2015. 34(1): p. 1-15.
370. Inci, I., Lung transplantation for emphysema. Annals of Translational Medicine, 2020. 8(21).

371. Hugh-Jones, P., et al., Lung transplantation in a patient with fibrosing alveolitis. Br Med J, 1971. 3(5771): p. 391-398.
372. Beyar, R., Challenges in organ transplantation. Rambam Maimonides medical journal, 2011. 2(2).
373. Guibert, E.E., et al., Organ preservation: current concepts and new strategies for the next decade. Transfusion Medicine and Hemotherapy, 2011. 38(2): p. 125-142.
374. Jing, L., et al., Organ preservation: from the past to the future. Acta Pharmacologica Sinica, 2018. 39(5): p. 845-857.
375. Tatum, R., et al., Machine perfusion of donor organs for transplantation. Artificial Organs, 2021. 45(7): p. 682-695.
376. Senior, M., Beating the organ clock. Nature Biotechnology, 2018. 36(6): p. 488-493.
377. Cypel, M., et al., Normothermic ex vivo perfusion prevents lung injury compared to extended cold preservation for transplantation. American Journal of Transplantation, 2009. 9(10): p. 2262-2269.
378. Halpern, S.E., et al., Lung transplantation after ex vivo lung perfusion versus static cold storage: an institutional cost analysis. American Journal of Transplantation, 2022. 22(2): p. 552-564.
379. Van Raemdonck, D., et al., Ex-vivo lung perfusion. Transplant International, 2015. 28(6): p. 643-656.
380. Cypel, M. and S. Keshavjee, Ex vivo lung perfusion. Operative Techniques in Thoracic and Cardiovascular Surgery, 2014. 19(4): p. 433-442.
381. Aboelnazar, N., et al., Ex-Vivo Lung Perfusion: From Bench to Bedside. Frontiers in Transplantology, 2016.
382. Aboelnazar, N.S., et al., Negative pressure ventilation decreases inflammation and lung edema during normothermic ex-vivo lung perfusion. The Journal of Heart and Lung Transplantation, 2018. 37(4): p. 520-530.
383. Buchko, M.T., et al., Clinical transplantation using negative pressure ventilation ex situ lung perfusion with extended criteria donor lungs. Nature Communications, 2020. 11(1): p. 5765.
384. Braithwaite, S.A., E. van Hooijdonk, and N.P. van der Kaaij, Ventilation during ex vivo lung perfusion, a review. Transplantation Reviews, 2023: p. 100762.
385. Vassilev, E. and M. McMichael, An overview of positive pressure ventilation. Journal of veterinary emergency and critical care, 2004. 14(1): p. 15-21.
386. Lai-Fook, S.J. and J.R. Rodarte, Pleural pressure distribution and its relationship to lung volume and interstitial pressure. Journal of Applied Physiology, 1991. 70(3): p. 967-978.
387. Dong, S.-J., et al., Lung resistance and elastance are different in ex vivo sheep lungs ventilated by positive and negative pressures. American Journal of Physiology-Lung Cellular and Molecular Physiology, 2022. 322(5): p. L673-L682.
388. Grasso, F., et al., Negative-pressure ventilation: better oxygenation and less lung injury. American journal of respiratory and critical care medicine, 2008. 177(4): p. 412-418.
389. Huang, L., et al., De Novo Design and Development of a Nutrient-Rich Perfusate for Ex Vivo Lung Perfusion with Cell Culture Models. International Journal of Molecular Sciences, 2023. 24(17): p. 13117.
390. Jeon, J.E., et al., Acellular ex vivo lung perfusate silences pro-inflammatory signaling in human lung endothelial and epithelial cells. Journal of Translational Medicine, 2023. 21(1): p. 729.

391. Steinmeyer, J., et al., Cellular and acellular ex vivo lung perfusion preserve functional lung ultrastructure in a large animal model: a stereological study. Respiratory research, 2018. 19: p. 1-15.
392. Chan, P.G., et al. Ex vivo lung perfusion: a review of research and clinical practices. in Seminars in Cardiothoracic and Vascular Anesthesia. 2020. SAGE Publications Sage CA: Los Angeles, CA.
393. Orens, J.B. and E.R. Garrity Jr, General overview of lung transplantation and review of organ allocation. Proceedings of the American Thoracic Society, 2009. 6(1): p. 13-19.
394. Studer, S., et al., Lung transplant outcomes: a review of survival, graft function, physiology, health-related quality of life and cost-effectiveness. European Respiratory Journal, 2004. 24(4): p. 674-685.
395. Wilkes, D.S., T.M. Egan, and H.Y. Reynolds, Lung transplantation: opportunities for research and clinical advancement. American journal of respiratory and critical care medicine, 2005. 172(8): p. 944-955.
396. Van Raemdonck, D., et al., Lung donor selection and management. Proceedings of the American Thoracic Society, 2009. 6(1): p. 28-38.
397. Pierre, A.F. and S. Keshavjee, Lung transplantation: donor and recipient critical care aspects. Current Opinion in Critical Care, 2005. 11(4): p. 339-344.
398. Saidi, R. and S.H. Kenari, Challenges of organ shortage for transplantation: solutions and opportunities. International journal of organ transplantation medicine, 2014. 5(3): p. 87.
399. Esmon, C.T., Basic mechanisms and pathogenesis of venous thrombosis. Blood reviews, 2009. 23(5): p. 225-229.
400. Hassan, M.I., A. Saxena, and F. Ahmad, Structure and function of von Willebrand factor. Blood coagulation & fibrinolysis, 2012. 23(1): p. 11-22.
401. Pappelbaum, K.I., et al., Ultralarge von Willebrand factor fibers mediate luminal Staphylococcus aureus adhesion to an intact endothelial cell layer under shear stress. Circulation, 2013. 128(1): p. 50-59.
402. Michels, A., et al., Investigating von Willebrand factor pathophysiology using a flow chamber model of von Willebrand factor-platelet string formation. JoVE (Journal of Visualized Experiments), 2017(126): p. e55917.
403. Collados, M.T., et al., Characterization of von Willebrand factor in primary pulmonary hypertension. Heart and vessels, 1999. 14: p. 246-252.
404. Lip, G.Y. and A. Blann, von Willebrand factor: a marker of endothelial dysfunction in vascular disorders? Cardiovascular research, 1997. 34(2): p. 255-265.
405. Akhtar, M., et al., The role of hypoxia-inducible factors in organ donation and transplantation: the current perspective and future opportunities. American Journal of Transplantation, 2014. 14(7): p. 1481-1487.
406. Mundt, H.M., et al., Optimized donor management and organ preservation before kidney transplantation. Transplant International, 2016. 29(9): p. 974-984.
407. Tozzi, M., et al., Impact of static cold storage VS hypothermic machine preservation on ischemic kidney graft: inflammatory cytokines and adhesion molecules as markers of ischemia/reperfusion tissue damage. Our preliminary results. International Journal of Surgery, 2013. 11: p. S110-S114.
408. Machuca, T.N. and M. Cypel, Ex vivo lung perfusion. Journal of thoracic disease, 2014. 6(8): p. 1054.

409. Spriggs, K.A., M. Bushell, and A.E. Willis, Translational regulation of gene expression during conditions of cell stress. Molecular cell, 2010. 40(2): p. 228-237.
410. Horioka, K., et al., Low temperature induces von-willebrand factor expression via increased early growth response 1 transcriptional activity in splenic sinusoidal endothelial cells. Biochemical and Biophysical Research Communications, 2020. 526(1): p. 239-245.
411. Brouland, J.-P., et al., In Vivo Regulation of von Willebrand Factor Synthesis: von Willebrand Factor Production in Endothelial Cells After Lung Transplantation Between Normal Pigs and von Willebrand Factor–Deficient Pigs. Arteriosclerosis, thrombosis, and vascular biology, 1999. 19(12): p. 3055-3062.