Age-related Comparison of Protein Composition in Serum and Platelet-Rich Fibrin (PRF)

by

Anand Parmar, DMD

BS, Michigan State University, 2012
DMD, Midwestern University, 2017

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This thesis was presented

by

Anand Parmar

It was defended on

June 6, 2024

and approved by

Charles Sfeir, DDS, PhD., Chair of Department of Periodontics

Camille Siqueira Nogueira, DDS, DSc, Assistant Professor Department of Periodontics

Thesis Advisor: Daniel Clark, DDS, MS, PhD, Assistant Professor Department of Periodontics

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ABSTRACT

Periodontal disease is one of several inflammatory disease that is found with a higher prevalence in the older adult populations. The pro-inflammatory cytokines IL-6 and TNF-α are two proteins that are found in higher concentrations in aging populations and may contribute to the pathogenesis of disease and healing alterations. Additionally, with healing alterations in this population we find a decrease in overall angiogenesis and regenerative potential with reduced delivery of growth factors. Without these growth factors present, conversion to healing phases maybe delayed or altered. Autologous blood products such as platelet-rich fibrin (PRF) are one way to improve wound healing and regeneration by delivering increased concentrations of growth factors. For the current study, growth factors VEGF and PDGF and pro-inflammatory cytokines IL-6 and TNF-α were chosen to be analyzed as a function of age in PRF and serum preparations. 43 participants (22 younger and 21 older adults) were enrolled in the study and 20mL of blood was collected for protein quantification. No statistically significant difference in the PRF and whole serum preparations of growth factors were noted in the older adult population. However, cytokine concentrations were statistically significantly higher after initial release 2hrs (day 1). Plasminogen was tested to further understand the degradation of fibrin clots and was significantly higher in both PRF and whole serum preparations in the older adult participant group. Further clinical studies evaluating the age-related differences in growth factors, cytokines and plasminogen and their relationship to wound healing outcomes would be of benefit in this field.

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Preface

Acknowledgment

I would like to thank all members of my MDS committee for not only facilitating this project to be started but also for the continued direction and support throughout this project. I would like to express immense gratitude to all members of the Clark Lab for their significant guidance and support throughout the initial planning phases, sample collections, protein quantifications all the way to the final analysis of data and thesis preparation. Your help has truly been appreciated and integral in the completion of this project, thank you!

1.0 Introduction

1.1 Periodontal Disease

Periodontal disease has been defined as a chronic multifactorial inflammatory disease associated with dental plaque biofilm and progressive destruction of the tooth supporting apparatus in a susceptible host (Papapanou et al. 2018). In periodontitis, the primary etiology in the form of plaque will cause an inflammatory host immune response leading to destruction of the hard and soft tissue components of the periodontal attachment apparatus including alveolar bone and both epithelial & connective tissue. Dysregulation of the host immune response may play an important role in the pathogenesis of periodontal disease. The initiation of the disease begins with the attachment of bacterial antigens to toll-like pattern recognition receptors on immune cells. This attachment activates signaling pathways leading to an increased immune and inflammatory response aimed to target the invading pathogens (Slots et al. 2017). Further, the bacteria mentioned in periodontitis are associated with a dysbiotic polymicrobial community leading to the stated inflammatory response. This unfortunately also leads to tissue destruction and a positive feedback loop with tissue breakdown products such as collagen peptides and heme products. These breakdown products become sources of amino acids and iron, further nourishing the dysbiotic microbiota in the gingival sulcus and promoting growth of pathogenic bacteria further causing imbalance and dysbiosis (Hajishengallis et al. 2020). This destructive process will present itself clinically as clinical attachment loss (CAL), periodontal pocketing with bleeding upon probing (BOP) along with other visual hallmark signs of inflammation. Radiographically, horizontal and/or vertical crestal alveolar bone loss will be present.

1.2 Prevalence of Periodontal Disease

In advanced stages of periodontitis, the cumulative damage of the supporting structures of teeth can lead to the loss of teeth. The complete loss of teeth in end stage disease or loss of teeth in greater numbers can cause patient disability due to reduced masticatory function which can lead to poor nutrition intake and impairment of quality of life (Bertolini & Clark 2023). It has been estimated by the Global Burden of Disease 2015 study that 11.2% of the world population is affected by severe periodontitis (Kassebaum et al. 2014). More recently, it has been re-estimated that the prevalence rate of severe periodontitis is 13.1% (Chen et al. 2021). In addition, it has been reported that age is a significant determinate of the clinical presentation of this chronic non-communicable disease (NCD). The contribution of periodontal pocketing and recession to the total attachment loss due to periodontitis differs with age (Sanz et al. 2020, Billings et al. 2017). A current systematic review and meta-analysis regarding the overall prevalence of periodontitis has reported levels to be 61.6% amongst adult population from 17 different countries and reported prevalence in elderly participants (≥65 years) to be higher (Trindade et al. 2023). More specifically in the United States, data from the National Health and Nutrition Examination Survey (NHANES) from 2009-2014 which utilized more accurate data collection than previous NHANES, showed 42% of adults 30 years or older have periodontitis and 7.8% having severe cases of periodontitis. In addition, adults 65 years or older exhibited the highest prevalence of periodontitis which continue to increase in prevalence with increasing age (Eke et al. 2018). As seen, periodontal disease affects a sizable portion of the aging US population leading to disabilities and reduction in quality of life.

1.3 Inflammatory Mediated Systemic Diseases

With an understanding of periodontal disease, we can further appreciate the inflammatory sequela of periodontal disease being associated with other inflammatory mediated systemic comorbidities that affect the older adult population including cardiovascular diseases, rheumatoid arthritis, cognitive impairment/Alzheimer's disease. From the findings of the 2019 consensus report organized by the European Federation of Periodontology (EFP) and the World Heart Federation (WHF), there is a strong positive association noted from epidemiological studies with periodontitis and coronary heart disease, which demonstrate patients with periodontal disease experiencing an increased risk of an incident atherosclerotic cardiovascular disease (ACVD) (Sanz et al. 2019). These updated findings were in addition to the conclusions from a 2012 joint workshop between the American Academy of Periodontics (AAP) and the European Federation of Periodontology (EFP) which stated, incident atherosclerotic cardiovascular disease (ACVD) and periodontitis has a biologic association involving circulating periodontal bacteria and an induction of systemic inflammation (Dietrich et al. 2013).

Rheumatoid arthritis (RA) is an autoimmune chronic inflammatory disease affecting joints leading to destruction, subsequent deformity and disability for patients. Although the etiology of rheumatoid arthritis has not been completely established, it is definitively known as a chronic inflammatory disease caused by multiple risk factors. An odds ratio range between 1.82-20.57 can be noted with a clear association between periodontitis and rheumatoid arthritis from several case-control studies (Gonzalez-Febles & Sanz 2021). These studies demonstrated patients with rheumatoid arthritis having higher prevalence of periodontal disease and subsequent tooth loss. Through further study and the understanding of the shared chronic inflammation processes that are present in both diseases, it has been established that the treatment of rheumatoid arthritis may

affect the periodontal status of patients with rheumatoid arthritis with a bidirectional relationship. Likewise, the treatment of periodontitis may have a positive and beneficial impact on the clinical and subclinical expression of rheumatoid arthritis which can be further understood with additional evidence through need further study (Gonzalez-Febles & Sanz 2021).

Following a proposed bidirectional relationship, Alzheimer's disease (AD) has been shown to share the effects of inflammation like periodontal disease. Systemic inflammation present in Alzheimer's disease contributes to neurodegeneration leading to a decline in memory, language, and cognitive skills in a slow and progressive manner (Liccardo et al. 2020). For Alzheimer's disease, periodontal pathological bacteria and their associated inflammatory response have been shown to cause local peripheral inflammation and damage which chronically will lead to neuroinflammation in the central nervous system (CNS). Thus, patients with periodontitis present at risk for the onset of Alzheimer's disease or worsening inflammatory related neurodegeneration of the central nervous system. (Dioguardi et al. 2020).

The inflammatory host response in humans is not only the key mechanism for periodontal disease, but also for other inflammatory conditions as seen. These stated chronic inflammatory conditions including periodontitis is often shown in aging populations leading to the release of circulating pro-inflammatory cytokines.

1.4 Inflammaging, Pro-Inflammatory Cytokines IL-6, TNF-α and Plasminogen (Plg)

Periodontal disease presents with an increase in circulating pro-inflammatory cytokines resulting from dysregulation of the host immune response which is found in greater prevalence in the aging population. The term inflammaging describes the age-related dysfunction of the host

immune response leading to elevated levels of pro-inflammatory cytokines. These changes lead to an increase in overall inflammation and increased prevalence of chronic inflammatory mediated systemic diseases (Clark et al. 2022). Several points about aging and increased systemic inflammation have been pointed out in the literature. These include the understanding that chronic diseases accelerate the ageing process in addition to being a result of aging and inflammaging. Dysfunction of the innate and adaptive immune response maybe caused by the age-related changes to the immune and hematopoietic stem cell function (Hajishengallis et al. 2020). With age, the chronic stimulation of the dysregulated host immune response leads to impaired immunity to pathogens and continued non-productive inflammation during the aging process further contributing to the effect of periodontitis including tissue destruction (Hajishengallis et al. 2020). Inflammation is considered to be part of the physiologic pathway of ageing shared with age-related diseases. Inflammation leads to alterations in stem cell regeneration, metabolism, proteostasis, macromolecular damage, stress and epigenetics (Franceschi et al. 2018).

Two commonly elevated pro-inflammatory cytokines in conditions of immune dysregulation or otherwise healthy aging adults are interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (Gordon et al. 2005). These pro-inflammatory cytokines are released by circulating monocyte derived macrophages during the host immune response to pathogens and initial injury to the tissue, leading to further inflammation, possible loss of tissue homeostasis and macrophage dysfunction (Gordon et al. 2005). During the initial phases of injury, the M1 (pro-inflammatory) macrophage phenotype is naturally expressed to aid in the defense against infection. The M2 (anti-inflammatory) macrophage phenotype is later expressed during the tissue and inflammation pro-resolution phase with release of ant-inflammatory cytokines such as interleukin-10 (IL-10) and additional growth factors to be discussed further in the upcoming growth factor subsection. During

periodontal disease the M1 macrophage phenotype is over expressed leading to prolonged pro-inflammatory processes leading to further tissue destruction and increased severity (Clark et al. 2022).

Interleukin-6 is a soluble mediator which is composed of 212 amino acids and has been gene mapped to chromosome 7p21 (Tanaka et al. 2014). In the presence of an initial stage of inflammation, IL-6 is released by pro-inflammatory M1 macrophages and travels to the liver to induce its effects on the production of proteins such as C-reactive protein (CRP), serum amyloid A (SAA), fibrinogen, and haptoglobin. With the production and accumulation of these proteins such as SAA, chronic inflammatory diseases may occur due to progressive deterioration of organs caused by the buildup. In addition, IL-6 promotes proliferation of immune cells including antibody production and CD4 T-cells (Tanaka et al. 2014). Increased levels of IL-6 are noted after the onset of periodontal disease, these increased levels lead to increases severity of destruction through further advancement of the pathways responsible for destruction of the attachment apparatus (Bertolini & Clark 2023).

Tumor necrosis factor-α (TNF-α) released by M1 macrophages is upregulated in inflammatory conditions, contributes to the pathogenesis of periodontal disease and is an overall marker for frailty in older adults. TNF-α has been gene mapped to chromosome 6p21.3 and exhibits several functional single nucleotide polymorphisms (SNP) (Ding et al. 2014). These variations in TNF-α are said to alter its normal function and thus leads to increased immunological dysfunction, promotion of chronic inflammation and overall increased risk for periodontitis with its direct cytotoxic effects (Li et al. 2020). Specifically, through activation of osteoclasts maturation, TNF-α promotes bone resorption and tissue destruction (Zhang et al. 2021). With these

prolonged and increased levels of inflammation, healing especially in the aging population is negatively affected.

Plasminogen (Plg) which has an essential role in fibrinolysis is a circulating glycoprotein synthesized primarily in the liver by hepatocytes and is found in plasma. Plasmin is the proteolytically active form of plasminogen, which is responsible for the removal of fibrin deposits and blood clots when physiologically activated as seen in figure 1 below. In addition to its role in fibrinolysis, plasminogen has several other important non-fibrinolytic functions related to the immune system, inflammation and wound healing (Yatsenko et al. 2023). Through plasminogen receptors, direct interaction with immune cells has been noted leading to activation of pro-inflammatory pathways with subsequent cytokine production. In contrast, plasminogen also has anti-inflammatory and immunosuppressive responses by regulating macrophage programing (Charithani & Medcalf 2021). With deficiency in plasminogen, diseases such as periodontitis are advanced. The accumulation of fibrin deposits without plasmin-mediated fibrinolysis leads to recruitment and retainment of more immune cells such as neutrophils. The antimicrobial defense functions of activated neutrophils become amplified leading to eventual immunopathology (Silva et al. 2023).

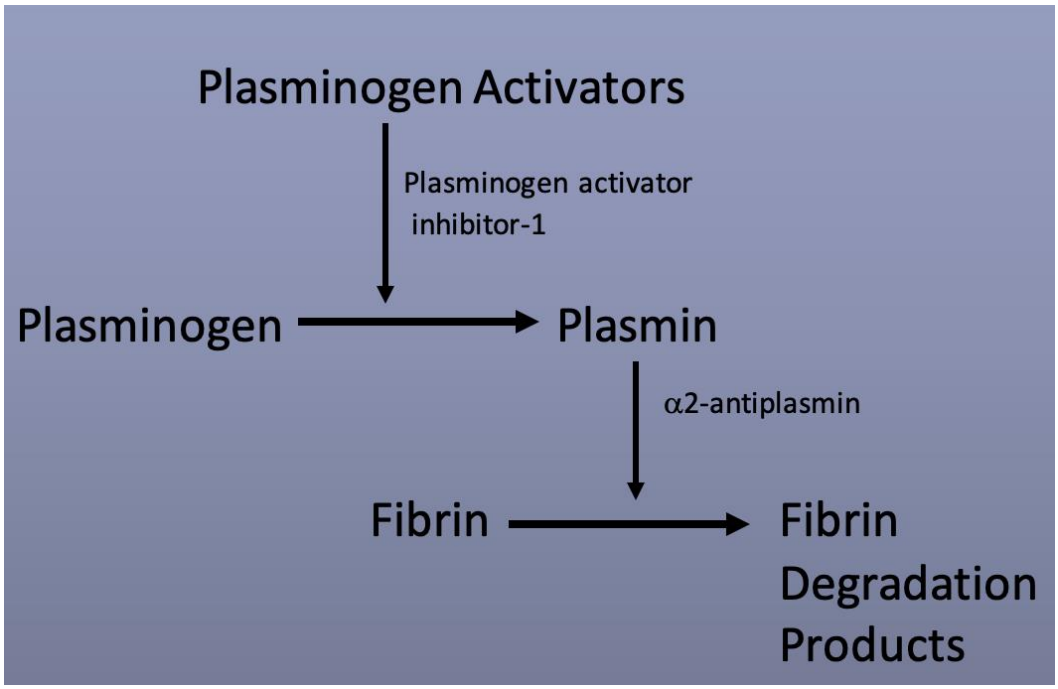

Figure 1: Plasminogen Pathway

1.5 Healing Alterations in Older Adults

There have been several identified possible causes of age-related alterations that lead to poor healing outcomes in older adults. Starting with the previously discussed pro-inflammatory M1 macrophage phenotype being chronically over expressed and delayed switching of M1 to the pro-resolution M2 macrophage phenotype found in age-related inflammatory disease states shown in figure 2. In the presence of this age-related continued M1 phenotype expression, continued increases in disease severity and osteolytic processes are noted with the lack of needed release of growth factors to promote subsequent healing phases (Clark et al. 2022). In addition, age-related alterations in noted from fracture healing studies in older adults gives light on the general expected

healing outcomes in this population. As previously mentioned, age-related decreases in mesenchymal stem cell function and quantities are noted which can lead to these immunomodulator's decreased ability to promote proliferation, differentiation and attenuation of inflammation which are needed for good healing outcomes (Hajishengallis et al. 2020). With age-related changes in vascularization, decreased perfusion and subsequent inadequate delivery of needed regenerative cells, nutrients and signaling molecules are emphasized. As a result, angiogenesis and overall regenerative potential is reduced (Clark et al. 2022). These stated age-related factors show the potential healing alterations in older adults and may highlight the need for additional growth factors to promote the conversion of pro-inflammatory states to healing states.

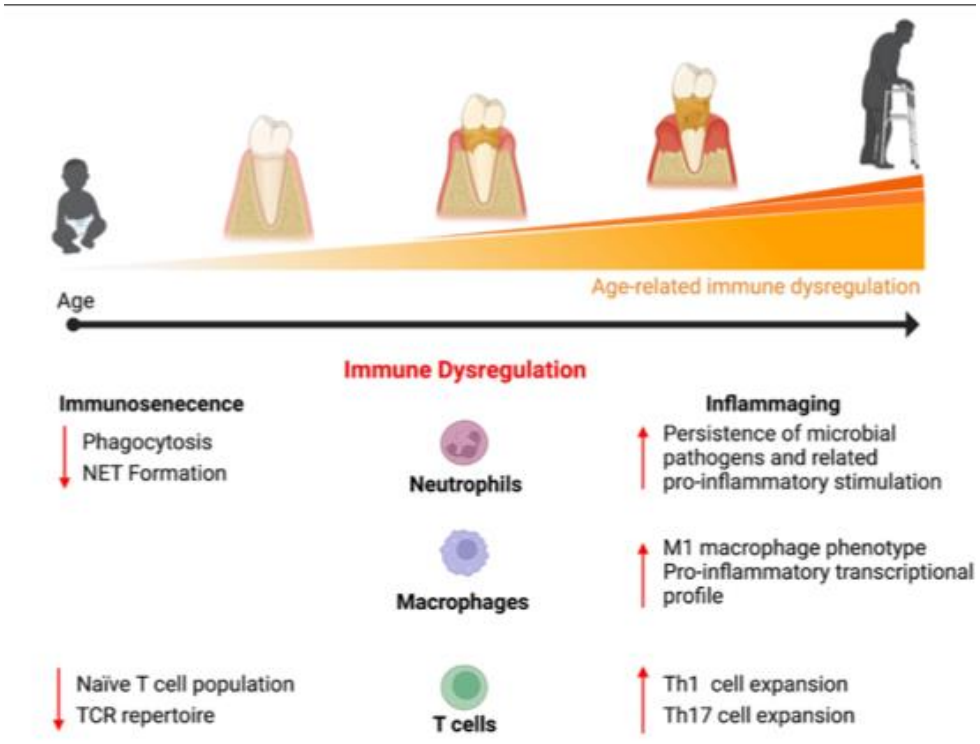

Figure 2: Age-related Immune Dysregulation. Clark et al. 2022

1.6 Growth Factors VEGF and PDGF

Vascular endothelial-derived growth factor (VEGF) gene mapped to 6p21.3 chromosome is one of the key growth factors involved in development, physiologic homeostasis, tissue/wound repair, and regeneration of the periodontal apparatus (Ren et al. 2021). Mainly targeting endothelial cells, VEGF facilitates vasculogenesis and angiogenesis which are crucial to the development of new blood vessel growth which serve maintain cell viability by bringing needed oxygen, nutrients and removal of catabolic waste products (Apte et al. 2019). This is achieved by VEGF being involved in cell proliferation, cell adhesion and chemotaxis of endothelial cells. In bone regeneration, VEGF released by osteoblasts aids in not only angiogenesis but also stimulates osteogenesis through regulation of osteogenic growth factors (Grosso et al. 2017). Similarly, platelet-derived growth factor (PDGF) released by platelets is an important protein involved in both soft and hard tissue homeostasis, repair and regeneration. Platelet-derived growth factor binds to specific cell-surface receptors and promotes cell migration (chemotaxis) and proliferation (mitogenesis) for gingival/periodontal ligament fibroblasts, cementoblasts and osteoblasts important for periodontal regeneration (Kaigler et al. 2011, Lynch et al. 1989).

1.7 Regeneration in Older Adults

As seen, older adults suffering from multiple chronic inflammatory mediated diseases including periodontitis are affected by dysregulation of the host immune response leading to continued pro-inflammatory states causing advanced destruction of the periodontal supporting structures of teeth which may lead to eventual tooth loss. With age-related healing alterations,

rehabilitating older adults who have suffered from advanced destruction becomes a challenging endeavor. Patients in these conditions often require not only disease resolution therapy but also regenerative surgeries to rebuild the destructed alveolar bone to provide returned masticatory function and improved quality of life.

1.8 Platelet-Rich Fibrin (PRF)

To aid in the regeneration of the periodontal attachment apparatus or ridge defects due to periodontitis/tooth loss, autologous blood-derived products (ABPs) such as platelet-rich fibrin (PRF) have been utilized and extensively studied in the literature. Initial uses were the first-generation fibrin adhesives and concentrated platelet-rich plasma (cPRP). Concentrated platelet-rich plasma was originally utilized for treatment of hemorrhages and for use topically in surgery. For clinical use, protocols using commercially available kits have been utilized with a 2-step centrifugation to concentrate platelets. During centrifugation, 3 distinct layers are separated including acellular plasma (PPP), platelet concentrate and red corpuscles. This platelet concentrate is isolated for both centrifugations to form the final liquid cPRP, which can be mixed with coagulating biomaterials such as bovine thrombin and calcium chloride at the time of use for gelling properties (Dohan et al. 2005). However, the multiple centrifugations and the addition of bovine thrombin add time, cost and complexity to the protocol. In addition, due to the nature the preparation, cytokines and growth factors that are released during the activation of platelets are not well incorporated into the fibrin matrix formed (after the addition of bovine thrombin after centrifugation) and are released largely in the first day (Miron et al. 2017). Ultimately, the amount

of cytokines and growth factors released depends on the centrifugation protocol and the preparation completed (Dohan et al. 2005, Leitner et al. 2006).

Plasma rich in growth factors (PRGF) was described by Anitua in 1999 and is included as one of the first concentrate protocols (Ehrenfest et al. 2008). Blood was collected in several small tubes which were subsequently centrifuged. Similarly to PRP, three distinct layers were noted after centrifugation: acellular plasma (PPP), a "buffy coat" layer and red corpuscles. The platelet poor plasma portion which was termed plasma poor in growth factors (PPGF) was pipetted out. The remaining plasma was termed plasma rich in growth factors (PRGF) and pipetted out with no exact measurements. It was then combined and mixed with 10% calcium chloride solution to form a fibrin matrix gel after 15-20 minutes used immediately after. Due to a non-precise protocol and changes in the collection method of the "buffy coat" this protocol suffered from reproducibility affecting its overall clinical benefit (Ehrenfest et al. 2008).

Platelet-rich fibrin is a second-generation platelet concentrate technology with a simplified and completely autologously derived preparation compared to previous generations including fibrin adhesives and concentrated platelet-rich plasma (cPRP) preparations. This updated and simplified technique no longer requires multiple centrifugations or the use of other biomaterials and naturally forms a fibrin clot (Ehrenfest et al. 2010). In order to understand the benefits of platelet-rich fibrin, it is important to understand what fibrin is and how it is derived in nature. Fibrin which is found in plasma and platelet α-granules is the activated form of the fibrinogen molecule and is involved in platelet aggregation during hemostasis (Dohan et al. 2005). When platelets are activated by tissue injury, activated blood cells or a foreign surface the enzymatic cascade of coagulation begins with the conversion of fibrinogen to fibrin by the action of thrombin. Through this conversion, the polymerization of a fibrin clot is noted with the transition of solution

to a space filling three-dimensional filamentous network with the cross-linking and branching of fibrin fibers (Weisel & Litvinov 2017).

The platelet-rich fibrin (PRF) protocol was developed by Choukroun et al. in 2001 to take advantage of the simple protocol for obtaining a blood derived product with the formation of the fibrin clot filled with activated platelets and the cytokines and growth factors they release. The originally described PRF protocol involves centrifuging of an autologous blood sample without anti-coagulant or gelling agents such as bovine thrombin. Activation of the platelets in contact with the tube wall is noted immediately and with centrifuging of the blood sample leading to the enzymatic cascade of coagulation. After centrifuging the blood sample, three distinct layers are formed top to bottom: supernatant serum (platelet poor plasma), platelet-rich fibrin and red corpuscles at the bottom. The clinical benefit of obtaining the platelet-rich fibrin clot is dependent on the speed of collection and the immediate initial transfer of the blood collection sample to the centrifuge (Dohan et al. 2005). In addition, deviating and not following the original protocol without reasoning may lead to a fibrin-poor clot with inadequate incorporated concentrations of cytokines and growth factors in the PRF-like clot, ultimately affecting the clinical benefit (Ehrenfest et al. 2010).

Embedded within the fibrin clot are not only the platelets but also the cytokines and growth factors that were released during platelet activation, which have been shown to be greater in release over time and in total numbers (Kobayashi et al. 2016). Found within are the previously discussed growth factors vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF), in addition to inflammatory cytokines such as the previously discussed interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). It is important to appreciate the concentration of both healing/repairing growth factors and pro-inflammatory cytokines involved in the control of

inflammation are to be found within the polymerized platelet-rich fibrin mesh network. The release of theses cytokines and growth factors at various times and quantities out of the fibrin mesh network in the form of exudate is well documented.

Since its initial description in 2001 by Choukroun et al., platelet-rich fibrin (PRF) has been slightly renamed by researchers in the field to leukocyte platelet-rich fibrin (L-PRF) using the (Intra-Spin L-PRF centrifuge) to reflect its natural high levels of leukocytes present in addition to previously noted platelets with associated cytokines and growth factors. Proponents of the L-PRF protocol identify that the Intra-Spin L-PRF centrifuge is the only CE marked and FDA cleared system (Ehrenfest et al. 2018). The original PRF protocol calls for a PC-02 table centrifuge and Process collection kits without anti-coagulant in 10mL glass-coated plastic tubes. Blood samples are then immediately centrifuged at 3000 rpm (force: 400g) for 10 minutes (Dohan et al. 2005). In slight contrast, the original L-PRF protocol calls for 9mL glass-coated plastic tubes with samples immediately centrifuged at 2700 rpm (force: 400g) for 12 minutes (Ehrenfest et al. 2018). This protocol spins blood samples 300 rpm less and for 2 minutes longer than the original PRF protocol discussed. With the identification of leukocytes being trapped in the matrix of the fibrin clot, we can appreciate the slow release of platelet growth factors not only from the fibrin clot but also from its release from the leukocytes themselves (Ehrenfest et al. 2008). In addition, the leukocyte immune cells play an important role in the healing process with their capability of directing and recruitment of cells important for this process (Fujioka-Kobayashi et al 2017).

It was later and further identified by Ghanaati et al. 2014 including Choukroun that with slower centrifugation speeds or (G-force), a higher concentration of leukocytes with a more even distribution could be noted within the fibrin clot matrix. This updated slower speed/lower G-force protocol named advanced platelet-rich fibrin (A-PRF), was also shown to release significantly

higher total platelet, protein and growth factors than the original PRF and L-PRF protocols (Kobayashi et al. 2016, Fujioka-Kobayashi et al. 2017). The findings of A-PRF containing higher concentrations of growth factors and leukocytes were also supported by Masuki et al. 2016 and several other studies in the literature. The updated centrifuge protocol involves immediately spinning the blood samples at 1500 rpm (force: 200g) for 14 minutes. With the increased concentrations of leukocytes, further influence on the differentiation of macrophages and release of growth factors has been noted which play an important role in tissue healing and regeneration.

With this understanding of slower and shorter centrifugation speeds lead to higher concentrations of platelets, growth factors and leukocytes, the A-PRF protocol was modified further to also take advantage of a liquid/injectable formulation and was termed injectable platelet-rich fibrin (i-PRF). An increased regenerative potential was noted over previous generation liquid PRP protocols and demonstrated longer and more gradual release of proteins. This updated formulation contains fibrinogen and thrombin (pre-conversion to fibrin) which allows a liquid formulation during its use (Miron et al. 2017, 2023). For the i-PRF protocol, 10mL glass-coated plastic tubes without anti-coagulants are used for blood collection and centrifugation at 700 rpm (force: 60g) for 3 minutes originally with a Duo Centrifuge. After centrifugation, the upper layer with a typical volume of 1-1.5mL is extracted as the i-PRF liquid concentrate which can be utilized alone or with other biomaterials and solidifying as a fibrin matrix clot after 10-15 minutes (Gollapudi et al. 2022). Improved handling properties of biomaterials such as bone graft have been noted during regenerative surgeries when the i-PRF protocol has been used and termed “sticky bone” when utilized. (Miron et al. 2023).

The most recent advances in platelet-rich fibrin address the longevity of the fibrin matrix to increase its benefit for use as barrier membranes in regenerative surgeries. Extended platelet-

rich fibrin (E-PRF) is obtained by heating the liquid acellular platelet-poor plasma (PPP) leading to denaturation of the present albumin which has been shown to extend the resorption properties to 4-6 months (Miron et al. 2023). As expected, present growth factors in the platelet poor plasma loose regenerative potential after heating and therefore after sufficient cooling the denatured membrane is mixed with PRF which is obtained from the "buffy coat" layer previously described under PRF protocol. Specifically, the E-PRF protocol involves blood collection in 10mL tubes without anti-coagulants and immediately centrifuged in horizontal centrifuge (Bio-PRF) for 8 minutes at a force of 700-2000g. 2mL of the acellular platelet poor plasma (PPP) is collected, heated to 75°C for 10 minutes to allow for albumin denaturation and then cooled with the Bio-Cool device for 2 minutes. During initial heating of the PPP, the remaining contents including the "buffy layer" were cooled then L-PRF was obtained from the sample using previously stated protocol. After preparation of the albumen gel and L-PRF, they are mixed together in two syringes about 10 times and then injected into the surgical site (Miron et al. 2023). As stated, its main benefit of extended resorption properties with present platelets, growth factors and leukocytes allow it to be used as a biologic filler lasting up to 6 months.

Another recent PRF area of study involves the difference in quality and quantity of L-PRF samples obtained through the traditional fixed-angle centrifuge versus PRF samples obtained through a horizontal centrifuge termed H-PRF. PRF obtained through horizontal centrifugation leads to less shear stress with less cell damage with even distribution and separation of layers with a more consistent g-force throughout the sample. Unlike traditional L-PRF centrifugation with uneven sloped separations with red blood cells noted throughout the layers, H-PRF results in up to 4 times as more cell concentrations (Miron et al. 2023).

Modifications and updates to protocols are currently being studied including changes in force, speed, time and temperature with the goal of obtaining an optimized PRF with maximum regenerative potential but also increasing its handing time with cooling and duration and resorption properties with heating and cooling protocols (Miron et al. 2023). Due to its autologous blood origins, the quantity and volume of platelet-rich fibrin is overall low (Dohan et al 2005). This may limit its use in large general surgery, however its benefit and use in periodontal surgery in including regenerative sites as supported by the American Academy of Periodontology best evidence consensus (Aliva-Ortiz et al. 2022).

As outlined by Miron et al. 2023, higher concentrations, improved growth factor release and increased cellular activity have been shown through updated studies with high RCF centrifugations. This is in contrast to previous slower speed centrifugation protocols including A-PRF and I-PRF. Termed concentrated PRF (C-PRF), this protocol concentrates cells within the "buffy coat" region to a 2-4x more than i-PRF while still maintaining a liquid form. These liquid formulations of PRF have been utilized in medicine for cartilage regeneration, osteo arthritis care with injection into joint space, wound care/healing, esthetic treatment including facial bio-filler injections, hair growth and drug delivery vehicles for small molecule medications and nanoparticles (Miron et al. 2023).

With the previously discussed increased prevalence of periodontal disease and subsequent tooth loss with alveolar bone destruction in the aging patient population, it has become increasingly more necessary to utilize regenerative surgeries for complex rehabilitation cases. Utilizing PRF's biologic properties, it has been shown to aid in the redirection macrophage polarization from pro-inflammatory M1 phenotype present in the dysregulation state of periodontal disease to a pro-resolution anti-inflammatory M2 phenotype which may be noted clinically by reduced post

operative swelling and pain after regenerative surgeries (Miron et al. 2023). However, platelet-rich fibrin's use in chronic dysregulation of inflammatory diseases and the complete understanding of its effects on immune cells must be continued in further research. With PRF's increased concentration of pro-inflammatory cells in addition to growth factors, its largely unknown what effect this may have on the local inflammatory environment. With this said, PRF may be a viable biologic additive to improve regenerative healing outcomes in older adults who currently or have suffered from destructive disease such as periodontitis.

Table 1: Platelet-rich Fibrin (PRF) Protocols

PREPARATION TYPE	**ROTATIONAL SPEED**		**TIME**	**TUBE COATING**
	(RPM)	(RCF)	(MIN)	
PRF	3000	400	10	Glass
LEUKOCYTE-PRF (L-PRF)	2700	700	12	Glass
ADVANCED-PRF (A-PRF)	1300	200	8-14	Glass
INJECTABLE-PRF (I-PRF)	700	60	3	Plastic

RPM: REVOLUTIONS PER MINUTE
RCF: RELATIVE CENTRIFUGAL FORCE

1.9 Specific Aims

The goal of this project was to use a blood collection protocol to test and observe any possible age-related differences in protein concentrations in both serum and PRF preparations from younger and older adult patients at the University of Pittsburgh School of Dental Medicine Department of Graduate Periodontics clinic. The specific proteins that were aimed to be analyzed were growth factors (VEGF & PDGF), inflammatory cytokines (IL-6 & TNF-α) and plasminogen (Plg).

We proposed the question, when compared to younger adult participants, would a lower serum concentration of growth factors with elevated levels of inflammatory cytokines in the older adult participant group be found, which have been seen to increase the prevalence of periodontitis and other inflammatory mediated systemic diseases (Eke et al. 2018). We further questioned whether platelet-rich fibrin (PRF) preparations would sufficiently concentrate growth factors in the older adult samples to make up any age-related differences noted in serum concentrations. In addition, we looked to identify possible increased concentrations of inflammatory cytokines and decreased concentrations of plasminogen in PRF preparations in the older adult samples when compared with younger adult samples. These younger adult samples may have exhibited lower serum concentration of inflammatory cytokines and higher serum concentrations of plasminogen when compared to older adult samples. Lastly, we aimed to identify changes in the release kinetics of the targeted growth factors, inflammatory cytokines and plasminogen proteins in the obtained PRF preparations.

Aim 1: To identify age-related differences in concentrations and release kinetics of growth factors (VEGF & PDGF), inflammatory cytokines (IL-6 & TNF-α) and plasminogen (Plg) in platelet-rich fibrin (PRF) preparations.

Aim 2: To identify age-related differences in whole serum concentrations of growth factors (VEGF & PDGF), inflammatory cytokines (IL-6 & TNF-α) and plasminogen (Plg).

Null Hypothesis 1: There is no statistically significant difference in platelet-rich fibrin (PRF) sample concentrations of tested growth factors (VEGF & PDGF) in the younger and older adult participant groups.

Null Hypothesis 2: There is no statistically significant difference between whole serum concentrations of tested growth factors (VEGF & PDGF) in the younger and older adult participant groups.

Null Hypothesis 3: There is no statistically significant difference in platelet-rich fibrin (PRF) sample concentrations of tested inflammatory cytokines (IL-6 & TNF-α) in the younger and older adult participant groups.

Null Hypothesis 4: There is no statistically significant difference between whole serum concentrations of tested inflammatory cytokines (IL-6 & TNF-α) in the younger and older adult participant groups.

Null Hypothesis 5: There is no statistically significant difference in platelet-rich fibrin (PRF) sample concentrations of Plasminogen (Plg) in the younger and older adult participant groups.

Null Hypothesis 6: There is no statistically significant difference between whole serum concentrations of Plasminogen (Plg) in the younger and older adult participant groups.

2.0 Material and Methods

2.1 Study Design

Participants from the University of Pittsburgh School of Dental Medicine Department of Graduate Periodontics were selected based on inclusion and exclusion criteria to participate in the study. To be eligible to participate in the study, patient's age had to fall within the following range: ≤ 35 years of age and ≥ 65 years of age. The selected age range was chosen based on the increased prevalence of periodontitis and inflammatory mediated systemic disease in patients aged 65 years of age and older (Eke et al. 2018). All participants were required to fill out a questionnaire which included their age, gender, BMI, current smoking status, use of blood thinners and diagnosis for periodontitis, hypertension, hypercholesterolemia, diabetes mellites. Self-reported medical diagnoses were confirmed with corresponding current patient prescriptions. No participants were excluded based on the presence of any systemic disease or condition in either of the specified age ranges. Informed consent was obtained by all eligible study participants after verbal discussion and review of written consent. Participant samples were collected from dates 12/12/2022 to 02/14/2024.

Inclusion Criteria:

- Patients were eligible to participate in the study if their age belonged in the following range: ≤ 35 years of age and ≥ 65 years of age.

Exclusion Criteria:

- Patients were ineligible to participate in the study if their age belonged in the following range 36-64 years of age.

2.2 Platelet-rich Fibrin (PRF) and Serum Preparation

From each younger and older adult participant, 20mL total of blood were collected into two separate 10mL sterile glass-coated plastic collection tubes without anticoagulant via peripheral venipuncture utilizing a standard butterfly needle. The two blood samples from each patient were be prepared at room temperature for following protocols using the Bio-PRF horizontal spin centrifuge.

PRF Sample: Collected blood sample was immediately centrifuged following Choukroun et al. standard protocol for the preparation of PRF, 3000 RPM (2660g) for 10 minutes. The PRF clot was removed from the collection tube.

Serum Sample: Collected blood sample was allowed to incubate motionless for 30 minutes to fully coagulate at room temperature. Once complete coagulation confirmed, sample was centrifuged for 10 minutes at 1000g. 2mL aliquot of supernatant (serum) was removed from the collection tube.

2.3 Sample Preparation

All samples were immediately transferred into 15mL plastic collection tubes, with 2mL of sterile Dulbecco's Modified Eagle Medium (DMEM) added only to PRF clot samples to submerge. All samples temporarily stored in ice until subsequent release kinetic testing and subsequent storage in laboratory freezer at -80°C prior to protein quantification analysis in set batches.

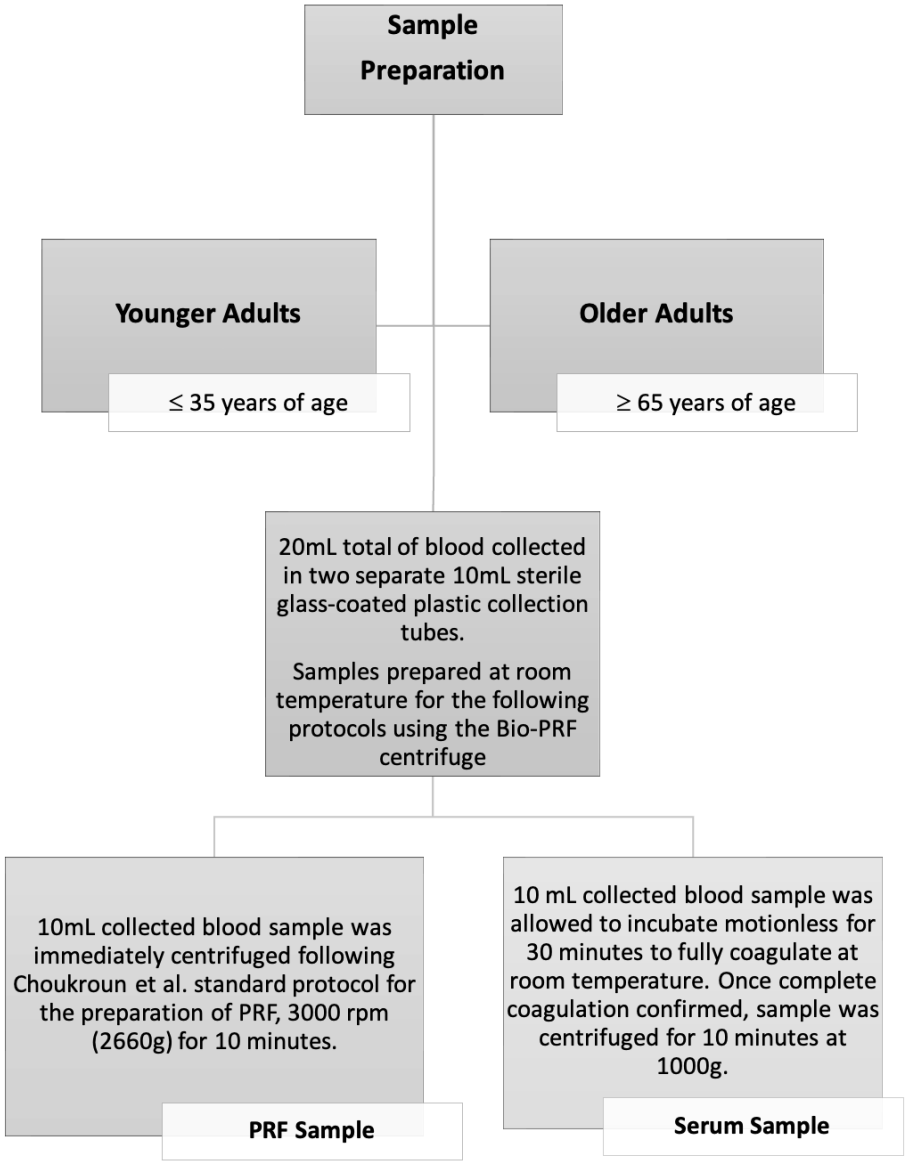

Figure 3: Platelet-rich Fibrin (PRF) and Serum Preparation

2.4 Platelet-rich Fibrin (PRF) Release Kinetics

Upon time for testing of the PRF samples, the release kinetics were evaluated at the following time points as also seen in Table 2 below:

2hrs (day 1), 24hrs (day 2), 48hrs (day 3), 96hrs (day 5), 168hrs (day 8)

Table 2: Platelet-rich Fibrin (PRF) Release Kinetics

PRF RELEASE TIME POINTS	HOURS	DAYS
	2	1
	24	2
	48	3
	96	5
	168	8

At the stated specified timepoints, transfer of the PRF clot was made to a new 15mL collection tube containing 2mL of fresh DMEM. The old collection tube was centrifuged at 500g for 5 minutes. A 325µl aliquot of the supernatant (serum) was collected and frozen at -80°C for later concentration analysis.

2.5 Multiplex Protein Quantification

Vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), interleukin-6 (IL-6) and tissue necrosis factor-α (TNF-α) protein quantification was conducted using the Millipore® Milliplex® Human Cytokine/Chemokine/Growth Factor Panel A Magnetic Bead Panel. The Milliplex ® ASSAY utilizes Luminex® xMAP® technology for microspheres with fluorescent-coated antibodies. After growth factor or cytokine is captured by the bead, a biotinylated detection antibody is formed, this reaction mixture is incubated with Streptavidin-PE conjugate. Samples are then analyzed with the Luminex® analyzer (Millipore® Milliplex® Human Cytokine/Chemokine/Growth Factor Panel A Magnetic Bead Panel User Manual).

2.6 Plasminogen (Plg) ELISA Quantification

Plasminogen protein quantification was conducted using the Biomatik® Human Plasminogen (Plg) ELISA Kit. Plasminogen ELISA is completed by utilizing a competitive enzyme immunoassay technique in which standard and sample concentrations of plasminogen are loaded to the pre-coated plasminogen microtiter plate wells with Horseradish Peroxidase (HRP) conjugated antibody preparation. A substrate solution is added to the wells which starts the competitive inhibition reaction between the pre-coated plasminogen and added standard plasminogen. The intensity of color is measured after color development, which is noted in an inverse relationship to the concentration to plasminogen (Biomatik® Human Plasminogen (Plg) ELISA Kit User Manual).

2.7 Statistical Analysis

Statistical analysis for the comparison of younger and older adult PRF and whole serum sample concentrations was completed by calculation of mean concentration values for each release time point 2hrs (day 1), 24hrs (day 2), 48hrs (day 3), 96hrs (day 5), 168hrs (day 8) for PRF and total mean concentration values for whole serum obtained from the raw data from the Multiplex protein quantification assay and plasminogen ELISA quantification tests. Using GraphPad software, an unpaired t-test was completed for the comparison of all the mean concentrations at the stated PRF release timepoints and total whole serum mean concentrations. The standard error of mean (SEM) and P-values were recorded for each comparison. PRISM software was used to enter all recorded data to fabricate PRF release kinetic mean concentration and whole serum mean concentration graphs.

3.0 Results

3.1 Characteristics of Study Participants

A total of 43 participants were enrolled into the current study after meeting the age-related inclusion criteria. There was a close to even distribution of younger to older adults with a total of 22 younger adults (≤ 35 years of age) and 21 older adults (≥ 65 years of age). The mean age for the younger adult participant group was 29.05 ± 4.66 and the mean age for the older adult participant group was 70.86 ± 3.45. As intended, a statistically extremely significant difference in the mean ages of both the younger and older adult groups was noted with a P-value of 0.0001. Within the 22 younger adult participant group, there were 12 males and 10 females. Within the 21 older adult participant group, there was 11 males and 10 females. A healthy younger adult (≤ 35 years of age) participant group was noted with no participants having a periodontitis, hypertension, hypercholesterolemia, or diabetes mellitus diagnoses (confirmed from self-reported participant medical history and prescribed medications). Additionally, no participants from this group had a self-reported smoking history and the group had a mean BMI of 23.38 ± 2.77 (healthy weight: 18.5-24.9). In the older adult participant group (≥ 65 years of age), out of 21 participants, 12 had periodontitis, 17 had hypertension, 15 had hypercholesterolemia, and 3 had diabetes mellitus diagnosis through same confirmation methods as younger adult participant group. 4/21 older adult participants reported smoking, 5/21 had prescribed blood thinners and the group had a mean BMI of 27.78 ± 3.52 (overweight: 25-29.9). There was a statistically significant difference in mean BMI between the younger and older adult groups with a P-value

of 0.0027. All participants enrolled into the study were considered successfully completed after 2 blood samples were obtained and processed.

Table 3: Characteristics of Study Participants

	YOUNGER ADULTS (N=22)	OLDER ADULTS (N=21)
AGE (MEAN ± SD)	29.05 ± 4.66	70.86 ± 3.45
GENDER (M/F)	12/10	11/10
PERIODONTITIS	0/22	12/21
HYPERTENSION	0/22	17/21
HYPERCHOLESTEROLEMIA	0/22	15/21
DIABETES MELLITUS	0/22	3/21
BMI (MEAN ± SD)	23.38 ± 2.77	27.78 ± 3.52
CURRENT SMOKER	0/22	4/21
BLOOD THINNERS	0/22	5/21

YOUNGER ADULTS = ≤ 35 YEARS OF AGE
OLDER ADULTS = ≥ 65 YEARS OF AGE

3.2 VEGF PRF Concentrations-Release Kinetics and Whole Serum Concentrations

Cumulative VEGF concentrations in pg/mL released from the collected PRF clot samples from younger and older adults were analyzed at 2hrs (day 1), 24hrs (day 2), 48hrs (day 3), 96hrs (day 5), 168hrs (day 8) time points with a calculated standard error of mean (SEM). At 2hrs (day 1) the mean concentration of VEGF released from the PRF sample in the younger adult participant group was 112.86 ± 12.98 pg/mL. The mean concentration of VEGF released from the older adult PRF samples was 95.27 ± 14.8 pg/mL. With a calculated P-value of 0.38, the difference in VEGF

released at 2hrs (day 1) was statistically not significantly different in the younger and older adult samples. At 24hrs (day 2) the mean concentration of VEGF released from the PRF sample in the younger adult participant group was 235.20 ± 22.90 pg/mL. The mean concentration of VEGF released from the older adult PRF samples was 182.61 ± 28.57 pg/mL. With a calculated P-value of 0.16, the difference in VEGF released at 24hrs (day 2) was statistically not significantly different in the younger and older adult samples. At 48hrs (day 3) the mean concentration of VEGF released from the PRF sample in the younger adult participant group was 292.26 ± 27.66 pg/mL. The mean concentration of VEGF released from the older adult PRF samples was 224.30 ± 35.16 pg/mL. With a calculated P-value of 0.14, the difference in VEGF released at 48hrs (day 3) was statistically not significantly different in the younger and older adult samples. At 96hrs (day 5) the mean concentration of VEGF released from the PRF sample in the younger adult participant group was 317.86 ± 29.75 pg/mL. The mean concentration of VEGF released from the older adult PRF samples was 248.1 ± 38.53 pg/mL. With a calculated P-value of 0.16, the difference in VEGF released at 96hrs (day 5) was statistically not significantly different in the younger and older adult samples. At 168hrs (day 8) the mean concentration of VEGF released from the PRF sample in the younger adult participant group was 329.63 ± 31.03 pg/mL. The mean concentration of VEGF released from the older adult PRF samples was 256.14 ± 39.44 pg/mL. With a calculated P-value of 0.15, the difference in VEGF released at 168hrs (day 8) was statistically not significantly different in the younger and older adult samples.

The total whole serum concentration of VEGF in the younger adult samples was 174.2 ± 31.37 pg/mL, whereas the total whole serum concentration of VEGF in the older adult samples was 197.91 ± 41.53 pg/mL. With a calculated P-value of 0.65, the difference in VEGF

concentration in whole serum was statistically not significantly different in the younger and older adult samples.

3.3 PDGF PRF Concentrations-Release Kinetics and Whole Serum Concentrations

Cumulative PDGF concentrations in pg/mL released from the collected PRF clot samples from younger and older adults were analyzed at 2hrs (day 1), 24hrs (day 2), 48hrs (day 3), 96hrs (day 5), 168hrs (day 8) time points with a calculated standard error of mean (SEM). At 2hrs (day 1) the mean concentration of PDGF released from the PRF sample in the younger adult participant group was 16316.21 ± 1140.94 pg/mL. The mean concentration of PDGF released from the older adult PRF samples was 15454.13 ± 1578.64 pg/mL. With a calculated P-value of 0.66, the difference in PDGF released at 2hrs (day 1) was statistically not significantly different in the younger and older adult samples. At 24hrs (day 2) the mean concentration of PDGF released from the PRF sample in the younger adult participant group was 36242.08 ± 2013.93 pg/mL. The mean concentration of PDGF released from the older adult PRF samples was 31853.91 ± 2842.08 pg/mL. With a calculated P-value of 0.22, the difference in PDGF released at 24hrs (day 2) was statistically not significantly different in the younger and older adult samples. At 48hrs (day 3) the mean concentration of PDGF released from the PRF sample in the younger adult participant group was 52838.96 ± 3030.78 pg/mL. The mean concentration of PDGF released from the older adult PRF samples was 46067.59 ± 3823.55 pg/mL. With a calculated P-value of 0.17, the difference in PDGF released at 48hrs (day 3) was statistically not significantly different in the younger and older adult samples. At 96hrs (day 5) the mean concentration of PDGF released from the PRF sample in

the younger adult participant group was 70641.11 ± 4418.15 pg/mL. The mean concentration of PDGF released from the older adult PRF samples was 61152.71 ± 4997.05 pg/mL. With a calculated P-value of 0.16, the difference in PDGF released at 96hrs (day 5) was statistically not significantly different in the younger and older adult samples. At 168hrs (day 8) the mean concentration of PDGF released from the PRF sample in the younger adult participant group was 93876.36 ± 9223.28 pg/mL. The mean concentration of PDGF released from the older adult PRF samples was 84506.90 ± 7858.67 pg/mL. With a calculated P-value of 0.44, the difference in PDGF released at 168hrs (day 8) was statistically not significantly different in the younger and older adult samples.

The total whole serum concentration of PDGF in the younger adult samples was 25814.01 ± 1390.39 pg/mL, whereas the total whole serum concentration of PDGF in the older adult samples was 23473.38 ± 2021.13 pg/mL. With a calculated P-value of 0.35, the difference in PDGF concentration in whole serum was statistically not significantly different in the younger and older adult samples.

3.4 IL-6 PRF Concentrations-Release Kinetics and Whole Serum Concentrations

Cumulative IL-6 concentrations in pg/mL released from the collected PRF clot samples from younger and older adults were analyzed at 2hrs (day 1), 24hrs (day 2), 48hrs (day 3), 96hrs (day 5), 168hrs (day 8) time points with a calculated standard error of mean (SEM). At 2hrs (day 1) the mean concentration of IL-6 released from the PRF sample in the younger adult participant group was 0.38 ± 0.29 pg/mL. The mean concentration of IL-6 released from the older adult PRF

samples was 1.77 ± 0.67 pg/mL. With a calculated P-value of 0.0655, the difference in IL-6 released at 2hrs (day 1) was statistically not quite significantly different in the younger and older adult samples. At 24hrs (day 2) the mean concentration of IL-6 released from the PRF sample in the younger adult participant group was 2164.13 ± 547.59 pg/mL. The mean concentration of IL-6 released from the older adult PRF samples was 5125.30 ± 1090.75 pg/mL. With a calculated P-value of 0.02, the concentration of IL-6 released at 24hrs (day 2) was statistically significantly higher in the older adult samples. At 48hrs (day 3) the mean concentration of IL-6 released from the PRF sample in the younger adult participant group was 6383.93 ± 1328.35 pg/mL. The mean concentration of IL-6 released from the older adult PRF samples was 12575.10 ± 2020.48 pg/mL. With a calculated P-value of 0.014, the concentration of IL-6 released at 48hrs (day 3) was statistically significantly higher in the older adult samples. At 96hrs (day 5) the mean concentration of IL-6 released from the PRF sample in the younger adult participant group was 8844.52 ± 1662.27 pg/mL. The mean concentration of IL-6 released from the older adult PRF samples was 17538.47 ± 38.53 pg/mL. With a calculated P-value of 0.0096, the concentration of IL-6 released at 96hrs (day 5) was statistically very significantly higher in the older adult samples. At 168hrs (day 8) the mean concentration of IL-6 released from the PRF sample in the younger adult participant group was 9725.08 ± 1768.03 pg/mL. The mean concentration of IL-6 released from the older adult PRF samples was 19239.44 ± 3017.41 pg/mL. With a calculated P-value of 0.0098 the concentration of IL-6 released at 168hrs (day 8) was statistically very significantly higher in the older adult samples.

The total whole serum concentration of IL-6 in the younger adult samples was 0.0 ± 0 pg/mL, whereas the total whole serum concentration of IL-6 in the older adult samples was 0.6310

± 0.28 pg/mL. With a calculated P-value of 0.037, the concentration of IL-6 released was statistically significantly higher in the older adult samples.

3.5 TNF-α PRF Concentrations-Release Kinetics and Whole Serum Concentrations

Cumulative TNF-α concentrations in pg/mL released from the collected PRF clot samples from younger and older adults were analyzed at 2hrs (day 1), 24hrs (day 2), 48hrs (day 3), 96hrs (day 5), 168hrs (day 8) time points with a calculated standard error of mean (SEM). At 2hrs (day 1) the mean concentration of TNF-α released from the PRF sample in the younger adult participant group was 7.57 ± 1.73 pg/mL. The mean concentration of TNF-α released from the older adult PRF samples was 7.51 ± 1.47 pg/mL. With a calculated P-value of 0.98, the difference in TNF-α released at 2hrs (day 1) was statistically not significantly different in the younger and older adult samples. At 24hrs (day 2) the mean concentration of TNF-α released from the PRF sample in the younger adult participant group was 158.23 ± 47.03 pg/mL. The mean concentration of TNF-α released from the older adult PRF samples was 602.09 ± 148.79 pg/mL. With a calculated P-value of 0.007, the concentration of TNF-α released at 24hrs (day 2) was statistically very significantly higher in the older adult samples. At 48hrs (day 3) the mean concentration of TNF-α released from the PRF sample in the younger adult participant group was 292.10 ± 70.22 pg/mL. The mean concentration of TNF-α released from the older adult PRF samples was 1353.76 ± 287.54 pg/mL. With a calculated P-value of 0.0009, the concentration of TNF-α released at 48hrs (day 3) was statistically very significantly higher in the older adult samples. At 96hrs (day 5) the mean concentration of TNF-α released from the PRF sample in the younger adult participant group was

519.66 ± 85.83 pg/mL. The mean concentration of TNF-α released from the older adult PRF samples was 1642.34 ± 325.87 pg/mL. With a calculated P-value of 0.0019, the concentration of TNF-α released at 96hrs (day 5) was statistically very significantly higher in the older adult samples. At 168hrs (day 8) the mean concentration of TNF-α released from the PRF sample in the younger adult participant group was 573.31 ± 92.23 pg/mL. The mean concentration of TNF-α released from the older adult PRF samples was 1724.46 ± 334.26 pg/mL. With a calculated P-value of 0.0020, the concentration of TNF-α released at 168hrs (day 8) was statistically significantly higher in the older adult samples.

The total whole serum concentration of TNF-α in the younger adult samples was 18.78 ± 2.45 pg/mL, whereas the total whole serum concentration of TNF-α in the older adult samples was 14.32 ± 1.95 pg/mL. With a calculated P-value of 0.160, the difference in TNF-α concentration in whole serum was statistically not significantly different in the younger and older adult samples.

3.6 Plg PRF Concentrations-Release Kinetics and Whole Serum Concentrations

Cumulative Plg concentrations in pg/mL released from the collected PRF clot samples from younger and older adults were analyzed at 2hrs (day 1), 24hrs (day 2), 48hrs (day 3), 96hrs (day 5), 168hrs (day 8) time points with a calculated standard error of mean (SEM). At 2hrs (day 1) the mean concentration of Plg released from the PRF sample in the younger adult participant group was 56144.77 ± 6953.51 pg/mL. The mean concentration of Plg released from the older adult PRF samples was 78403.32 ± 7978.5 pg/mL. With a calculated P-value of 0.044, the concentration of Plg released at 2hrs (day 1) was statistically significantly higher in the older adult

samples. At 24hrs (day 2) the mean concentration of Plg released from the PRF sample in the younger adult participant group was 91346.86 ± 10529.56 pg/mL. The mean concentration of Plg released from the older adult PRF samples was 125956.6 ± 11135.29 pg/mL. With a calculated P-value of 0.0314, the concentration of Plg released at 24hrs (day 2) was statistically significantly higher in the older adult samples. At 48hrs (day 3) the mean concentration of Plg released from the PRF sample in the younger adult participant group was 118744.02 ± 12505.24 pg/mL. The mean concentration of Plg released from the older adult PRF samples was 168038.68 ± 13668.61 pg/mL. With a calculated P-value of 0.0124, the concentration of Plg released at 48hrs (day 3) was statistically significantly higher in the older adult samples. At 96hrs (day 5) the mean concentration of Plg released from the PRF sample in the younger adult participant group was 158814.92 ± 16250.97 pg/mL. The mean concentration of Plg released from the older adult PRF samples was 241011.52 ± 19768.13 pg/mL. With a calculated P-value of 0.0031, the concentration of Plg released at 96hrs (day 5) was statistically significantly higher in the older adult samples. At 168hrs (day 8) the mean concentration of Plg released from the PRF sample in the younger adult participant group was 201241.86 ± 20572.84 pg/mL. The mean concentration of Plg released from the older adult PRF samples was 291237.81 ± 24769.19 pg/mL. With a calculated P-value of 0.009, the concentration of Plg released at 168hrs (day 8) was statistically significantly higher in the older adult samples.

The total whole serum concentration of Plg in the younger adult samples was 111884.53 ± 7253.66 pg/mL, whereas the total whole serum concentration of Plg in the older adult samples was 167573.85 ± 16555.76 pg/mL. With a calculated P-value of 0.0044, the concentration of Plg in whole serum was statistically significantly higher in the older adult samples.

Table 4: VEGF, PDGF, IL-6, TNF-α & Plasminogen PRF Cumulative and WS Concentrations

RELEASE TIME POINTS (hrs)	YOUNGER ADULTS MEAN ± SEM (pg/mL)	OLDER ADULTS MEAN ± SEM (pg/mL)	P-VALUE
VEGF PRF			
2	112.86 ± 12.98	95.27 ± 14.8	0.38
24	235.20 ± 22.90	182.61 ± 28.57	0.16
48	292.26 ± 27.66	224.30 ± 35.16	0.14
96	317.86 ± 29.75	248.1 ± 38.53	0.16
168	329.63 ± 31.03	256.14 ± 39.4	0.15
VEGF WS	174.2 ± 31.37	197.91 ± 41.53	0.65
PDGF PRF			
2	16316.21 ± 1140.9	15454.13 ± 1578.64	0.66
24	36242.08 ± 2013.93	31853.91 ± 2842.08	0.22
48	52838.96 ± 3030.78	46067.59 ± 3823.55	0.17
96	70641.11 ± 4418.15	61152.71 ± 4997.05	0.16
168	93876.36 ± 9223.2	84506.90 ± 7858.67	0.44
PDGF WS	25814.01 ± 1390.39	23473.38 ± 2021.13	0.35
IL-6 PRF			
2	0.38 ± 0.29	1.77 ± 0.67	0.0655
24	2164.13 ± 547.59	5125.30 ± 1090.75	0.02
48	6383.93 ± 1328.35	12575.10 ± 2020.48	0.014
96	8844.52 ± 1662.27	17538.47 ± 38.53	0.0096
168	9725.08 ± 1768.03	19239.44 ± 3017.41	0.0098
IL-6 WS	0.0 ± 0	0.6310 ± 0.28	0.037
TNF-α PRF			
2	7.57 ± 1.73	7.51 ± 1.4	0.98
24	158.23 ± 47.03	602.09 ± 148.79	0.007
48	292.10 ± 70.22	1353.76 ± 287.54	0.0009
96	519.66 ± 85.83	1642.34 ± 325.8	0.0019
168	573.31 ± 92.23	1724.46 ± 334.26	0.0020
TNF-α WS	18.78 ± 2.45	14.32 ± 1.95	0.160
Plg PRF			
2	56144.77 ± 6953.51	78403.32 ± 7978.51	0.044
24	91346.86 ± 10529.56	125956.6 ± 11135.29	0.0314
48	118744.02 ± 12505.24	168038.68 ± 13668.61	0.0124
96	158814.92 ± 16250.97	241011.52 ± 19768.13	0.0031
168	201241.86 ± 20572.84	291237.81 ± 24769.19	0.009
Plg WS	111884.53 ± 7253.66	167573.85 ± 16555.76	0.0044

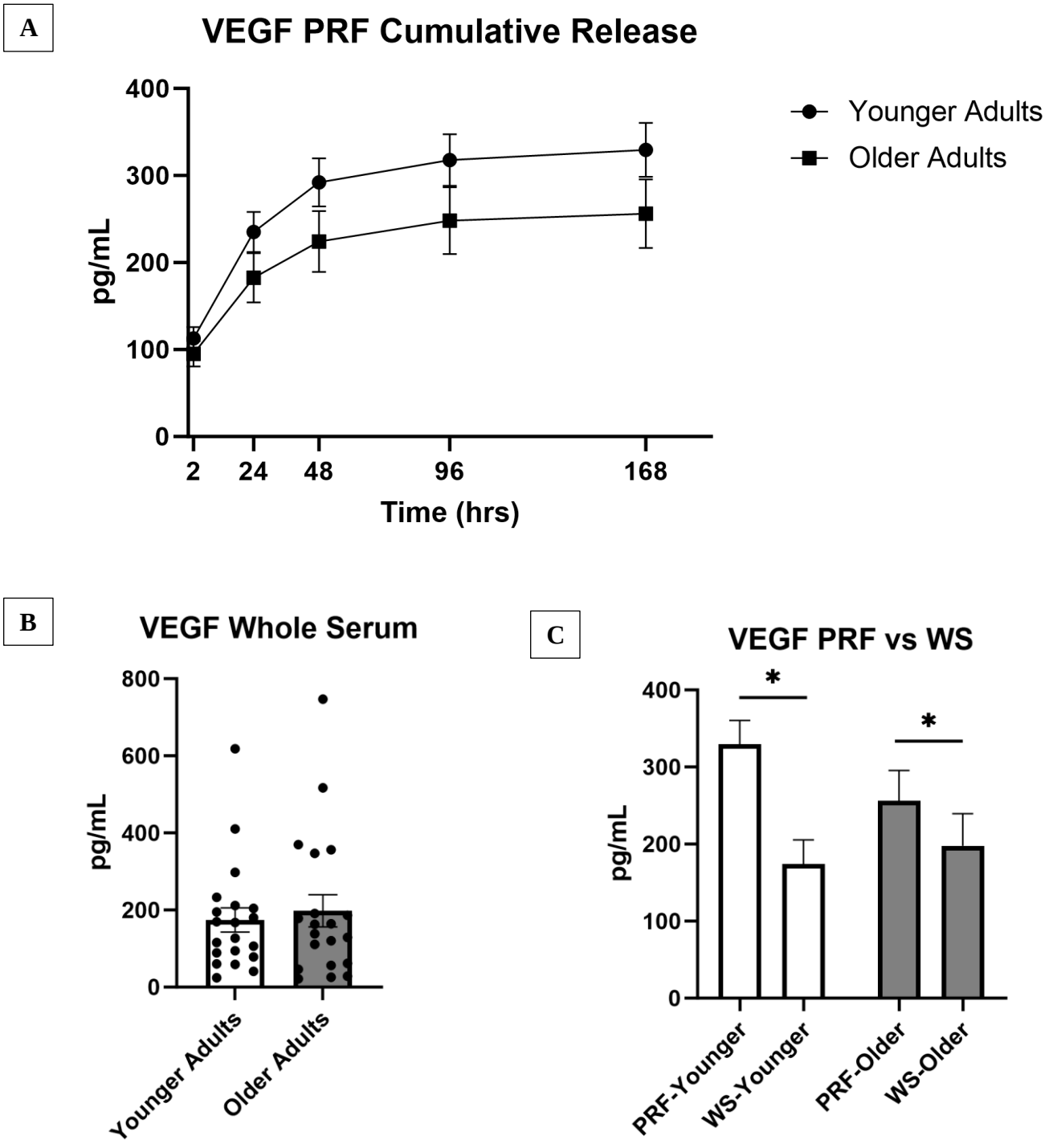

Figure 4: VEGF PRF and Whole Serum Release Concentrations (mean ± SEM)

A: VEGF PRF Cumulative Release
B: VEGF Whole Serum
C: VEGF PRF vs WS- * P-value = < 0.05

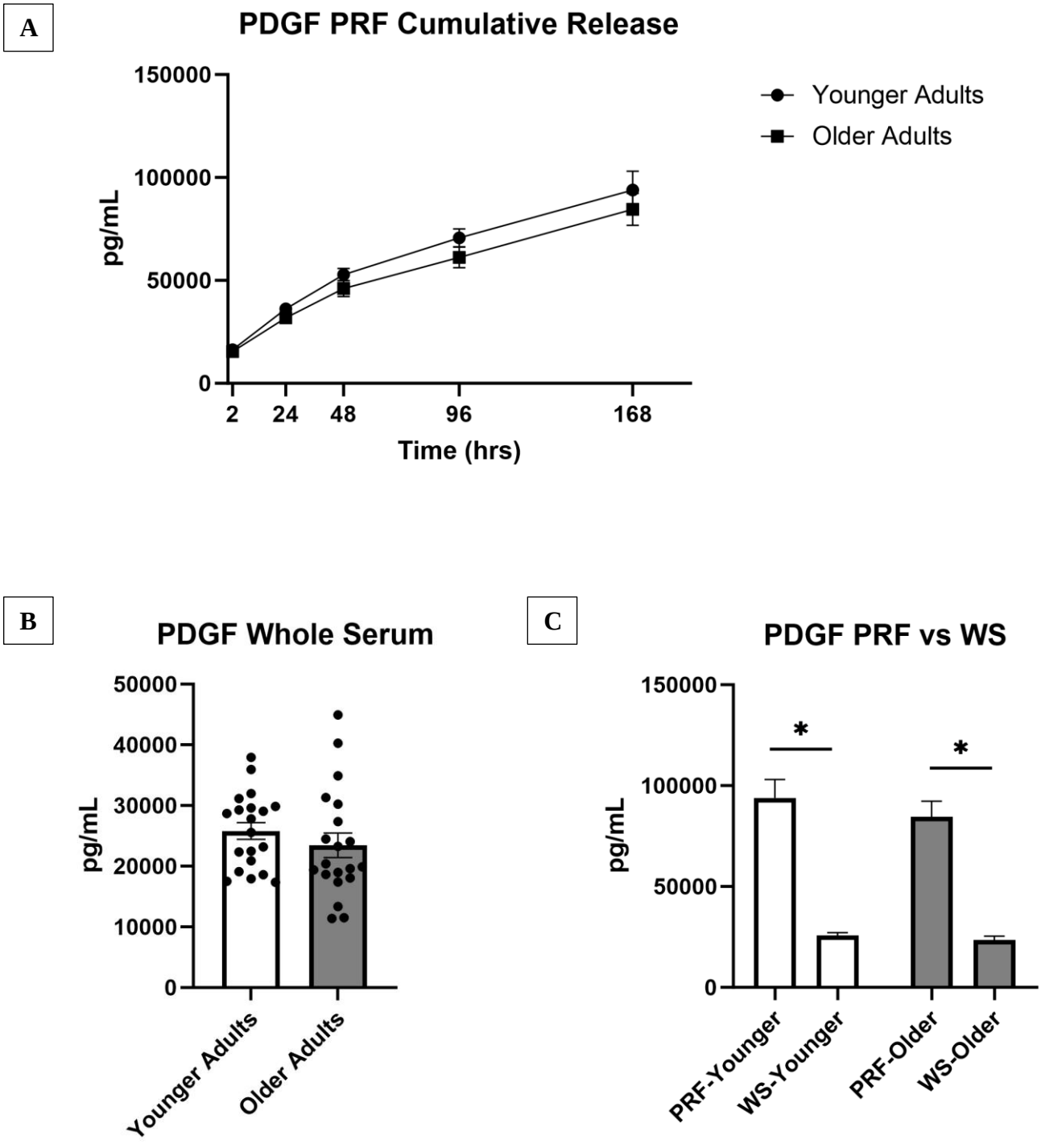

Figure 5: PDGF PRF and Whole Serum Release Concentrations (mean ± SEM)

A: PDGF PRF Cumulative Release
B: PDGF Whole Serum
C: PDGF PRF vs WS- * P-value = < 0.05

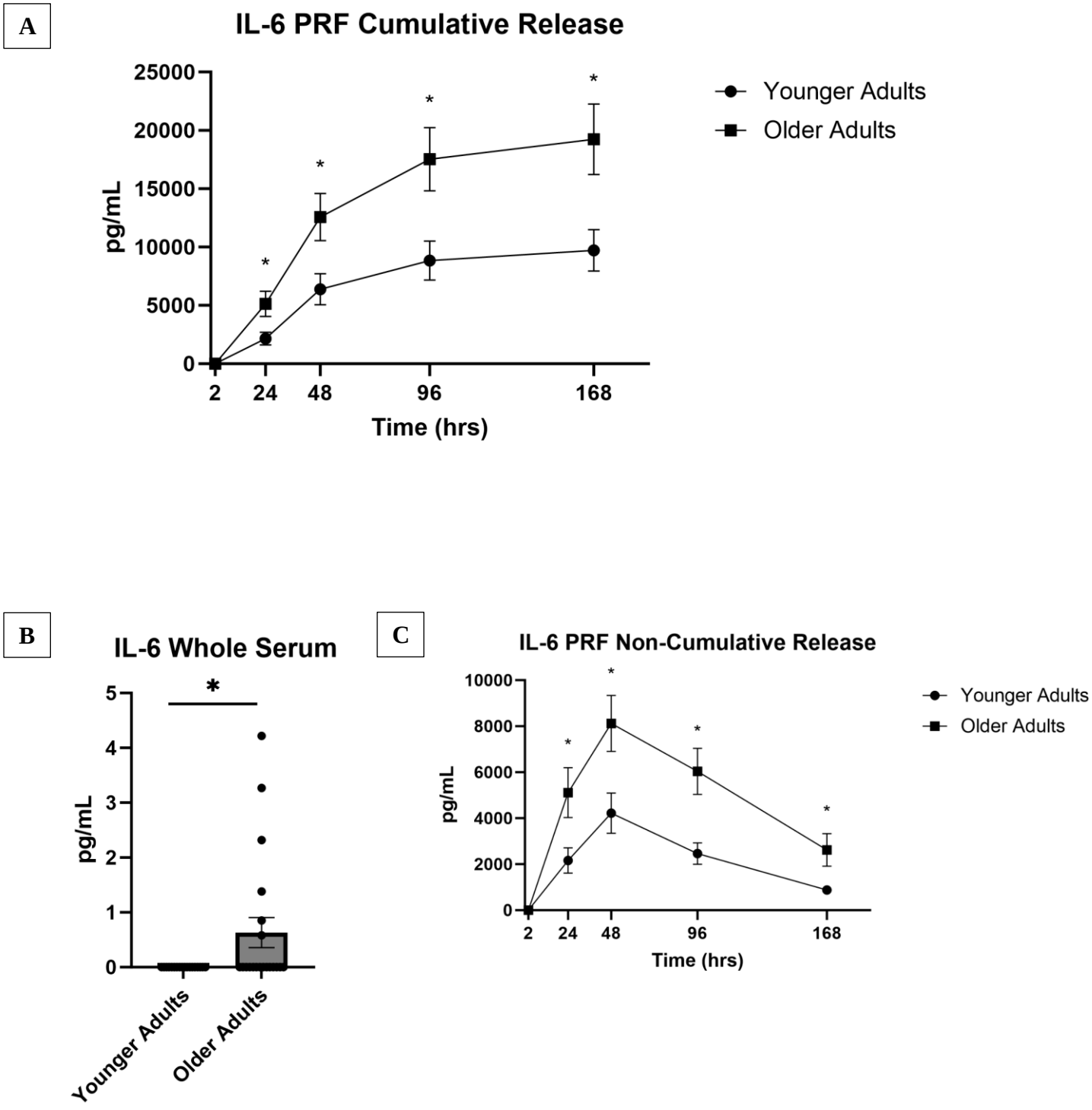

Figure 6: Il-6 PRF and Whole Serum Release Concentrations (mean ± SEM)

A: IL-6 PRF Cumulative Release * P-value = < 0.05
B: IL-6 Whole Serum * P-value = < 0.05
C: IL-6 PRF Non-Cumulative Release * P-value = < 0.05

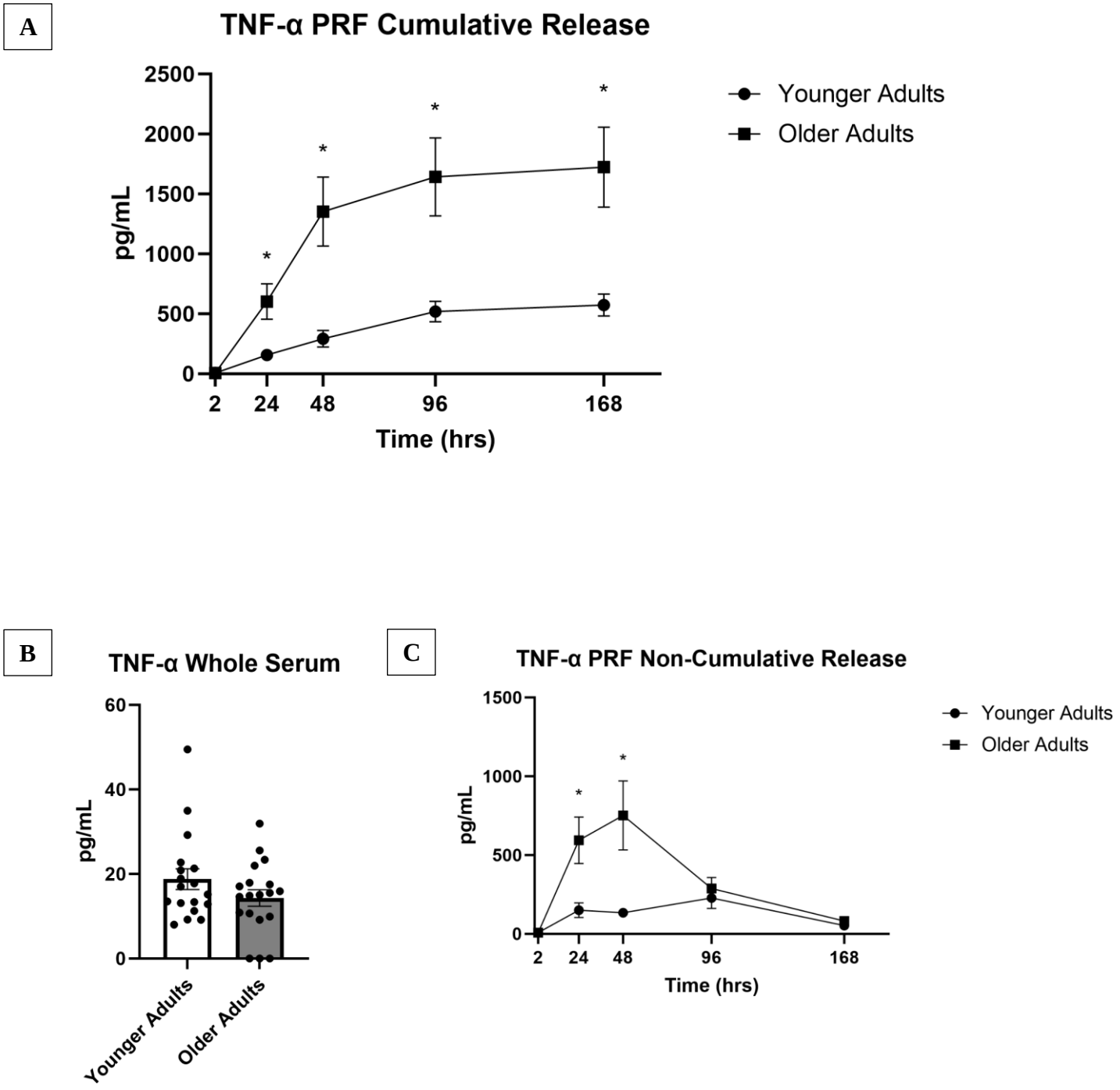

Figure 7: TNF-α PRF and Whole Serum Release Concentrations (mean ± SEM)

A: TNF-α PRF Cumulative Release * P-value = < 0.05
B: TNF-α Whole Serum
C: TNF-α PRF Non-Cumulative Release * P-value = < 0.05

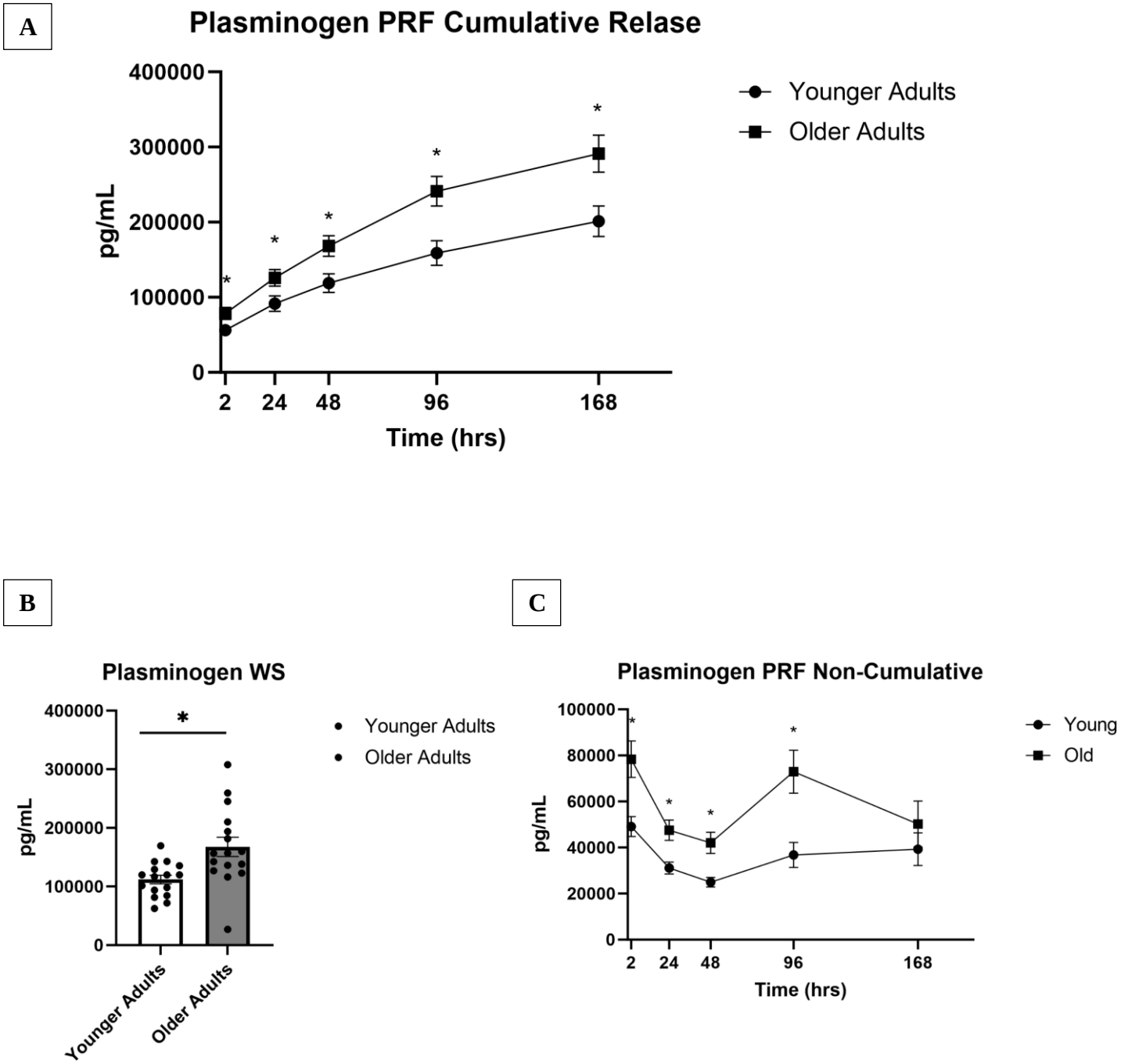

Figure 8: Plasminogen PRF and Whole Serum Release Concentrations (mean ± SEM)

A: Plg PRF Cumulative Release * P-value = < 0.05
B: Plg Whole Serum * P-value = < 0.05
C: Plg PRF Non-Cumulative * P-value = < 0.05

4.0 Discussion

An overall challenge in comparing two different participant groups in this current study was obtaining a differing population based of one criteria point such as age but having similar enough other characteristics to be effectively compared. With age being the primary planned comparison amongst the two different groups, a statistically significant difference in mean age values was noted as seen table 3. An overall healthy younger adult participant group was noted with no current diagnosis for periodontitis or any systemic disease/condition such as hypertension, hypercholesterolemia, or diabetes mellitus. In addition, no current smokers or participants in the younger adult group was prescribed blood thinners. In contrast, a majority in the older adult participant group had a periodontitis (12/21), hypertension (17/21) and hypercholesterolemia (15/21) diagnosis. 3/21 older adults had a diabetes mellitus diagnosis, 4/21 older adults were current smokers and 5/21 older adults were currently prescribed blood thinners. In addition, there was a statistically significant difference in BMI between the two age groups, with the older adult participant group having higher BMI scores. With these differences amongst the two groups, one may dispute the comparison. However, the current study's older adult participant group exhibits medical characteristics which is representative of the ≥65 years old adult population. 12/21 or 57% of this participant group had a periodontitis diagnosis which within the expected 42-62% range described in the literature for adults within this age range (Trinidade et al. 2023, Eke et al. 2018). In addition, the 74.5% of adults 60 and over with hypertension reported in the literature is also representative of the current studies 17/21 or 81% hypertension diagnosis amongst the ≥65 years old adult participant group. (Ostchega et al. 2020). With this and although possible, finding a non-

representative smaller healthy adult population for comparison was out of the scope of the current study.

Many findings were made from the protein quantification analysis completed from both PRF and whole serum samples from the younger and older adult participant groups. For the protein growth factors analyzed in this study (VEGF & PDGF), although no statistically significant difference was noted between the younger and older adult VEGF and PDGF PRF sample concentrations, increasing concentrations were noted at all recorded time points. This finding demonstrated a substantiated and continued release of growth factors from the PRF samples up to the final recorded 168hrs (day 8) time point in both groups. At the PRF release time point 2hrs (day 1), both VEGF and PDGF exhibited the closest younger and older adult mean concentrations which were also greater than 0 pg/mL (112.86 ± 12.98 pg/mL and 95.27 ± 14.8 pg/mL, 16316.21 ± 1140.94 pg/mL and 15454.13 ± 1578.64 pg/mL, respectively). This finding demonstrated an immediate release of the growth factors VEGF and PDGF from the PRF samples. VEGF whole serum concentrations were higher in older adult samples than younger adults and PDGF whole serum concentrations were higher in younger adult samples than older adults (174.2 ± 31.37 pg/mL vs. 197.91 ± 41.53 pg/mL, 25814.01 ± 1390.39 pg/mL vs. 23473.38 ± 2021.13 pg/mL, respectively). However, similarly to PRF sample concentrations there was no statistically significant difference in the VEGF and PDGF whole serum samples concentrations between the younger and adult samples showing no deficit of growth factors found in the older adult study population. For both VEGF and PDGF, the total cumulative concentration at the final recorded 168hrs (day 8) time point was higher than the recorded total whole serum concentration for both the younger and older adult groups. This result demonstrates growth factors in both age groups can be successfully increased in concentration and as stated released over at least a recorded 168hr

(8 day) period of time. Based on these stated findings, our null hypothesis 1 holds true with there being no statistically significant difference in platelet-rich fibrin (PRF) sample concentrations of tested growth factors (VEGF & PDGF) in the younger and older adult participant groups. In addition, our null hypothesis 2 holds true with there being no statistically significant difference between whole serum concentrations of tested growth factors (VEGF & PDGF) in the younger and older adult participant groups.

Many differences in findings were noted when the pro-inflammatory cytokine protein quantification analysis was completed for IL-6 and TNF-α. Although IL-6 was found in higher concentration in the PRF preparations for older adult participants (0.38 ± 0.29 pg/mL vs. 1.77 ± 0.67 pg/mL) at release time point 2hrs (day 1), there was no statistically significant difference in concentrations from the younger and older adult populations. Similarly, at release time point 2hrs (day 1) for TNF-α there was no statistically significant difference in concentrations from the younger and older adult populations and was found to be slightly higher in the younger adult participant group (7.57 ± 1.73 vs 7.51 ± 1.47 pg/mL). These very low concentrations of IL-6 and TNF-α at timepoint 2hrs (day 1) demonstrated a delayed release of these pro-inflammatory cytokines from the PRF sample. This finding is in contrast to the noted higher concentration and immediate release of both growth factors in the current study, VEGF and PDGF. As clearly shown in figure 6C, the peak release of pro-inflammatory cytokine IL-6 was at 48hrs which is in line with the middle of the late inflammatory stages of periodontal wound healing. Similarly shown in figure 7C, pro-inflammatory cytokine TNF-α release was highest at 96hrs (day 5) which falls within the late inflammatory stage of periodontal wound healing (Polimeni et al. 2006). Furthermore, the M1 pro-inflammatory macrophage phenotype is largely expressed during the inflammatory wound healing stages from days 3-5. M1 pro-inflammatory macrophages release the pro-inflammatory

cytokines IL-6 and TNF-α which supports the PRF non-cumulative release findings (Chen et al. 2022). For both IL-6 and TNF-α at all subsequent release time points 24hrs (day 2), 48hrs (day 3), 96hrs (day 5), 168hrs (day 8), there was a statistically significant concentration difference between the younger and older adult PRF. As compared to younger adults, older adult participants had significantly higher concentrations of both IL-6 and TNF-α for these stated release time points. Increasing concentrations were noted at all recorded time points demonstrating continued substantiated release of pro-inflammatory cytokines from the PRF samples up to the final recorded 168hrs (day 8) time point in both groups. Whole serum concentrations of IL-6 were statistically significantly different between the younger and older adult groups (0.0 pg/mL and 0.6310 ± 0.28 pg/mL). These findings are supported in the literature with young adults having peak IL-6 serum concentrations between 0-1 pg/mL and with older adults having higher concentrations (Alberro et al. 2021). In order to help quantify young adult IL-6 serum samples to be greater than 0 pg/mL, a higher sensitivity assay could be used in future studies. In contrast, for TNF-α there was no significant difference between the younger and older adult whole serum concentrations (18.78 ± 2.45 pg/mL and 14.32 ± 1.95 pg/mL, respectively). For both IL-6 and TNF-α, the total cumulative concentration at the final recorded 168hrs (day 8) time point was significantly higher than the recorded total whole serum concentration for both the younger and older adult groups. This result demonstrates the increased concentration of pro-inflammatory cytokines found within the PRF sample which are also released over at least a recorded 168hr (8 day) period of time. A clinical trial would need to be conducted in order to determine the effect of this increased concentration of pro-inflammatory cytokines and their extended release to a surgical site. Although unknown from this study, providing increased concentrations of pro-inflammatory cytokines to a surgical site past the initial inflammatory wound healing phases may delay or alter healing. Additionally, increased

concentrations of pro-inflammatory cytokines may prevent conversion M1 pro-inflammatory macrophage to M2 pro-resolution macrophage phenotypes. Based on these overall stated findings, our null hypothesis 3 holds true for only release time point 2hrs (day 1) and can be rejected for all other release time points demonstrating there is a statistically significant difference in platelet-rich fibrin (PRF) sample concentrations of tested inflammatory cytokines (IL-6 & TNF-α) in the younger and older adult participant groups. In addition, our null hypothesis 4 holds true for TNF-α with there being no statistically significant difference between whole serum concentrations of tested inflammatory cytokine in the younger and older adult participant groups. Finally, our null hypothesis 4 can be rejected for IL-6 with there being a statistically significant difference between the whole serum concentrations between the younger and older adult groups.

With an understanding of the release kinetics and expected concentrations of growth factors and cytokines within platelet-rich fibrin preparations we wanted to further question breakdown of the fibrin clot and how it may impact the initial release and in addition the duration of release of the proteins evaluated in this study. As previously discussed, plasminogen and its active form plasmin are part of one of the pathways involved in the break fibrin clots into fibrin degradation products. In the PRF preparations within this study, it was noted that plasminogen levels were statistically significantly higher at all release time points 24hrs (day 2), 48hrs (day 3), 96hrs (day 5), 168hrs (day 8) for older adults. Release of plasminogen from the PRF samples was noted up until at least the last recorded time point of 168hrs (days 8), with younger and older adults showing a similar cumulative release pattern. Interestingly though, plasminogen's release from the PRF samples exhibited a similar unique non-cumulative release pattern from both the younger and older adult groups. Plasminogen was noted to release in higher concentrations at time point 2hrs (day 1) with a decrease in concentrations noted at both 24hrs (day 2) and 48hrs (day 3) time points.

Plasminogen concentrations then peaked to the second highest concentration at 96hrs (day 5) before dropping again at 168hrs (day 8) time point. With this secondary peak of plasminogen at 96hrs (day 5) after peak cytokine release at 48-96hrs, it maybe hypothesized that in part, the increased release of plasminogen could be due to the stimulation from the released cytokines. In addition, interestingly not only do fibrin clots increase immune cell quantity and function, but the breakdown products of fibrin also increase the immune response. Similarly to the PRF preparations, the higher plasminogen concentration levels noted in the older adult whole serum group was statistically significant. Increased circulating level of plasminogen maybe due to in part to related pathways both upstream and downstream to the plasminogen to plasmin pathway.

These overall findings demonstrate that older adult participants in this current study have statistically no significant difference in tested growth factors in whole serum from younger adult participants. With these similar levels of growth factors found in whole serum, both participant groups could benefit from the concentration of these growth factors in platelet-rich fibrin (PRF) preparations whose difference in concentrations have been shown not to be statistically significant. The similar growth factor concentration levels found in PRF preparations in the older adult participant group could be of benefit for use in regenerative surgery after this population has suffered from higher prevalence levels of systemic disease and increased destruction of alveolar bone from periodontitis as shown in this current study.

Although, not within the scope of the present study it may be important to understand the change in PRF research. Over the period of time since it was first described by Choukroun et al., PRF and its protocols have evolved to increase its clinical efficacy for use in healthcare. This has been realized through further understanding of its preparation to concentrate and deliver more immune cells and proteins in a sustained beneficial way. Today, the most up to date protocols for

PRF including injectable PRF (i-PRF), extended PRF (E-PRF), concentrated PRF (C-PRF) have been utilized not only in the intra oral cavity for regenerative surgeries but also extra-orally in the medicine field for a variety of indications including cartilage regeneration, osteo arthritis care with injection into joint space, wound care/healing, esthetic treatment including facial bio-filler injections, hair growth and drug delivery vehicles for small molecule medications and nanoparticles (Miron et al. 2023).

The need for further study in the general PRF literature is pronounced due to the changing protocols and understanding of the factors that may limit or increase its clinical benefit. These factors include protocol changes to increased and decreased speed or RCF, fixed angle or horizontal centrifugation, utilization of heating and cooling methods to change PRF's physical properties and substantivity, and hydrophobic/hydrophilic test tube design to cause or reduce activation of platelets thus altering the state of PRF. Numerous further projects involving PRF at the University of Pittsburgh School of Dental Medicine Department of Periodontics can be of meaningful benefit for the continued understanding of its benefits and increased utilization for patients of the university clinic. Many projects can be developed to assess and validate the current PRF protocols including PRF with liquid formulations and extended absorption properties with the same young and older adult populations present to continue to identify age-related differences in concentrations as are described in the current study with the traditional PRF protocol. In addition, analysis of the concentration of pro-inflammatory cytokines maybe noted with protocols which may alter is clinical benefit. Continuation of this topic will allow for a more complete understanding of the benefits and efficacy of growth factor concentration in PRF for use in a variety of regenerative surgeries in patients affected from age-related diseases.

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