Title

Differences in Concentration of Growth Factor Proteins in Platelet Rich Fibrin among Diabetics and Non-Diabetics: An Exploratory Study

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Differences in Concentration of Growth Factor Proteins in Platelet Rich Fibrin among Diabetics and Non-Diabetics: An Exploratory Study

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Differences in Concentration of Growth Factor Proteins in Platelet Rich Fibrin among Diabetics and Non-Diabetics: An Exploratory Study

Neil Patel

Abstract

Initially introduced in 2000 by Choukroun et al., platelet-rich fibrin (PRF) emerged as an autologous blood concentrate prepared through centrifugation, finding utility in both dentistry and medicine.[3] PRF is widely used in dental procedures such as soft tissue grafting, ridge preservation, bone grafting, and sinus lift procedures, exhibiting notable advantages in wound healing, clinical outcomes, and handling.[3-7] Studies have revealed that L-PRF releases higher concentrations of growth factors like PDGF-BB and VEGF. Notably, there is a lack of research on the impact of type 1 or type 2 diabetes on growth factor content in L-PRF, despite diabetes being one of the three risk factors for periodontal disease, adversely affecting wound healing, immune cell function, and regenerative outcomes in periodontal surgeries.[25;30;33] This case-control pilot study is aimed at comparing the growth factor concentrations in L-PRF samples obtained from healthy non-diabetic individuals and diabetic individuals. Three 10ml vacuum glass tubes of autologous venous blood were collected per patient, comprising five healthy non-diabetic patients and five diabetic patients. Observed findings from the enzyme-linked immunosorbent assays (ELISA) demonstrated no statistical difference in the growth factor concentrations for PDGF-BB and VEGF when comparing diabetes and healthy subjects. However, trends showed decreased levels of PDGF-BB and VEGF with age in patients with diabetes. These results suggest that there may be no additional benefit with the adjunctive usage of L-PRF in periodontal surgery patients with diabetes.

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Introduction

Platelet-rich plasma (PRP) was introduced for adjunctive use in dental surgery to be an autologous source of growth factors[1]. This material was prepared by using a centrifuge to produce an autologous blood concentrate that could be used in various treatment modalities in dentistry. Due to a lack of evidence, PRP was not widely utilized; this led to the development of a second-generation concentrate known as platelet-rich fibrin (PRF) was developed to have an increased potential for growth factor release as well as a longer duration of action.[2-4] Past studies have shown the materials' clinical benefits when used to facilitate improved handling of biomaterials, wound healing, and treatment outcomes.[5-8]

PRF is an autologous blood product that is isolated via centrifugation of blood samples taken intravenously from patients. The process of centrifugation acts as an initiator leading to platelet aggregation, activation, and subsequent release of numerous signals and proteins varying from cytokines to growth factors.[9-11] Choukran et al. introduced the protocol for producing L-PRF. [3;12] This is done by collecting a 10mL sample of blood in a glass-coated plastic tube which is spun at 2700 rpm for 12 mins.[12-14] L-PRF is named because of its unique property to produce a higher concentration of leukocytes. Microscopy and ELISA showed that L-PRF outperformed other forms of PRF and released higher concentrations of growth factors including platelet-derived growth factor BB (PDGF-BB) and Vascular Endothelial growth factor (VEGF).[15] Therefore, it was postulated that the usage of PRF in dental surgery greatly promoted wound healing, angiogenesis, and immune cell recruitment.

L-PRF has become widely adopted in the field of periodontics and dental surgery. These applications range from being used as a membrane for improved wound healing and for use as

and autologous fibrin glue that can be used with other biomaterials like particulate allograft or xenograft to enhance handling, cytokine release, and growth factor concentrations.[16-17] The combination of L-PRF and particulate graft is commonly referred to as "sticky bone" and is made by using a plastic tube for centrifugation where the L-PRF is isolated as a viscous fluid instead of a solid.[18-19] Beyond dentistry, PRF has found numerous applications in various fields of medicine. Grecu et al. studied the potential benefits of using PRF in orthopedic injuries. They found that the recovery time for PRF-treated patients was reduced to 16.6 days compared to 22.3 days for those solely given anti-inflammatory agents.[20] In addition, L-PRF has also become a treatment option to help accelerate the healing of patients suffering from diabetic wounds. A systematic review from Wong et al. found that usage of PRF was associated with an increased rate of wound healing.[21]

A previous study by Kim et al. investigated the efficacy of L-PRF based on its handling and peak efficacy and showed that the peak levels of growth factors were observed 90 minutes after centrifugation of the sample.[22] Another study by Tamraz et al. investigated the effects of cigarette smoking on the levels of growth factors found in L-PRF samples. This study showed a significant increase in the levels of VEGF and PDGF-BB in smoking patients compared to non-smokers.[23] Just as smoking is a recognized risk factor for periodontitis and a known contributor to systemic inflammation, diabetes also falls into this category. Consequently, the discoveries made by Tamraz et al. have prompted further exploration into how other chronic inflammatory conditions, such as diabetes, impact the growth factor content of L-PRF.[23]

Diabetes mellitus is a disease of metabolic origin that occurs because of elevated glucose levels in the blood. There are 2 major forms of diabetes found among patients. Type 1 diabetes mellitus occurs due to a defect in insulin secretion whereas type 2 diabetes occurs due to defects in

insulin action or effect. The pathogenesis for both types is distinct with various etiologies, clinical presentations, and methods of treatment.[24]

There are 3 major risk factors found in periodontal disease: smoking, diabetes, and pathogenic bacteria. Diabetes specifically has a large-scale impact on wound healing and the immune response. Diabetes will have a direct effect on the function of a person's immune cells. It also affects a person's osteoblast and fibroblast function, inhibiting bone turnover and tissue attachment. Patients with diabetes also exhibit the formation of advanced glycation end products (AGEs). This glycosylation process leads to increased thickness in the vascular basement membranes and impedes the transport of ions and molecules across this cell layer. Lastly, these AGEs will also bind and activate the AGE-binding macrophage receptor (RAGE) increasing the production of pro-inflammatory cytokines like IL-1β and TNF-α.[25]

In the field of periodontics, diabetes heavily influences treatment decisions and outcomes. Previous studies have shown that diabetes is a risk factor for periodontitis and a potential contributor to implant failure. [26-31] A recent meta-analysis assessing the incidence of diabetes and periodontitis found that diabetes increased the chances of a patient developing or having periodontitis by 86%.[32] The influence of diabetes on periodontitis is abundantly clear, and diabetics with an HbA1c over 6.99% are categorized into a higher risk group categorized as Grade C according to the most recent diagnostic system from the World Workshop in 2018.[33]

This exploratory study was designed to investigate if L-PRF is an effective adjunctive material to help facilitate superior treatment outcomes in patients with diabetes who would normally be at risk for impaired and delayed healing. Specifically, the study aims to evaluate the potential difference in growth factor levels of PDGF-BB and VEGF. Evaluation of these levels may help clinicians better treat those with impaired wound healing or altered immune responses during

dental surgery. Although limited in its potential clinical impact, studies such as this and the ones completed by Kim et al. and Tamraz et al. help to set the tone for future investigation and treatment protocols. Similar to Tamraz et al.'s study on L-PRF and smoking, this study was designed to look at the potential effect diabetes has on growth factor levels without possible confounding variables, such as history of smoking, systemic disease, or systemic medications.[22-23]

Given the impairment in healing outcomes observed in diabetics, looking for adjunctive treatment options that can help improve healing and promote successful outcomes is pivotal. Therefore, looking into the possible benefits of L-PRF for this population is crucial. We hypothesized that there may be notable differences in protein/growth factor content in diabetics, leading to the possible clinical benefits of using L-PRF to facilitate superior surgical and treatment outcomes. The goal of this exploratory study was to evaluate the growth factor concentrations of PDGF-BB and VEGF in L-PRF obtained from diabetic and non-diabetic patients from the Division of Periodontology at UCSF School of Dentistry.

Materials and Methods

Patient Population and Enrollment

Subjects from the UCSF School of Dentistry Division of Periodontology were screened and enrolled in the study. The screening process included an extensive review of medical history (HbA1c, systemic diseases, medications, smoking status, etc.) and the signing of an IRB-approved consent form (IRB #20-33191). Those who enrolled were divided into 2 subgroups: Non-Diabetic (Healthy subjects) and Diabetic groups. Non-diabetic subjects were included when they were over 18 years of age, had no previous diagnosis of diabetes or pre-diabetes, had no previous history of smoking/vaping, and had no reported use of anti-coagulants. The diabetes/test

subjects were included when they were over 18 years of age and had either type 1 or type 2 diabetes with an HbA1c of at least 7.0% and had no previous history of smoking/vaping and no reported use of anti-coagulants. Five of the subjects (aged 29-72) were assigned to the healthy group as they had no history of diabetes (Type 1 or Type 2) or smoking (tobacco, marijuana, or recreational drugs). The other five subjects (aged (49-86) were assigned to the diabetes group as they reported HbA1c levels ranging from 7.0% - 9.2%, and no history of smoking (tobacco, marijuana, or recreational drugs). All enrolled subjects who agreed to participate in the study signed the consent form. The collected data was organized by date of blood draw and diabetes status, Healthy (H) compared to Diabetic (D) (**Table 1**).

Sample Extraction

After consent forms were reviewed and signed, venous blood was collected from either the right or the left antecubital vein using 3 tubes (two 10 mL sterile glass tubes and one 10 mL sterile plastic tube: Nalgene Cryoware). The collected blood samples were then centrifuged at 2700 revolutions per minute for 12 minutes with an Intra-Spin centrifuge [Intra-Lock International, Birmingham, AL].[14] Once the centrifugation was completed, one of the glass tubes with L-PRF was labeled and set aside to undergo the 90-minute waiting period before being transferred to sterile cryotubes for storage in a -80°C freezer.[22] The other tubes were subsequently used for the subject's ongoing periodontal procedure that day. Once all the test samples were collected, they were transported in biohazard-compliant dry ice containers to the laboratory where the samples underwent freezing (-80°C) and processing for subsequent Invitrogen ELISA Growth factor analysis for PDGF-BB and VEGF.

Sample Preparation

To maintain the sterility and purity of the sample, all handling and preparation of the L-PRF was completed in a biosafety level 2 plus laminar flow hood. The L-PRF samples were removed from the -80°C freezer and semi-thawed to room temperature after which the samples were safely removed from the cryotubes. The desired L-PRF resides between an upper acellular plasma layer and a lower red blood cell layer. This layer is then separated from the acellular and blood cell layers and stored and the other layers are disposed of into the correct biosafety container. From the semi-thawed state, the L-PRF membrane was separated into 3 equal pieces with a sterile blade. Each isolated L-PRF membrane was then analyzed via the protein ELISA assays to check for the desired growth factors. (**Figure 1**)

ELISA Analyses of Growth Factors

The enzyme-linked immunosorbent assay (ELISA) was carried out following the protocol provided in the Invitrogen [Thermo Fisher Scientific, Waltham, MA, USA] kit for growth factor proteins PDGF-BB and VEGF. The reagents were prepared initially with buffer concentrates brought to room temperature, starting with a wash buffer. To prepare the wash buffer, 50 ml of the concentrate (20x) was transferred into a 1000 ml graduated cylinder and diluted to 1000 ml with deionized water, then thoroughly mixed. This wash buffer was then stored at 2°C. Additionally, assay buffer (5 ml) was poured into a 100 ml graduated cylinder and brought to a final volume of 100 ml with distilled water, before being stored at 2°C. The biotin-conjugate was diluted 1:100 with assay buffer in a sterile plastic tube, while the streptavidin-HRP was also diluted 1:100 with assay buffer. The test protein was reconstituted with assay buffer for 15 minutes and mixed for homogenous solubilization (4000 pg/ml). Dilution of the external standard involved 7 tubes for standard points, labeled S1-7. A 2-fold serial dilution was prepared

by pipetting 250 µl of assay buffer into each tube. Then, 250 µl of reconstituted standard (concentration 4000 pg/ml) was added to the first tube (S1) and mixed, resulting in a concentration of S1 = 2000 pg/ml. This process was repeated for subsequent tubes, creating a standard curve with six additional points (**Figure 2**).[23]

The test protocol commenced by pre-diluting the sample 1:10 with assay buffer, following the formula of 20 µl sample and 180 µl assay buffer. Microwell strips were then removed from the holder and placed in a foil-covered bag with desiccant at 2°C. Each microwell strip underwent two washes with 400 µl wash buffer, with aspiration performed between washes. Excess wash buffer was removed from the strips by tapping them on an absorbent pad. Following this, 100 µl of standard dilutions (S1-7) were pipetted into the standard wells, while 100 µl of assay buffer was added in duplicate to the blank wells, and 50 µl of assay buffer was added to the sample wells. Subsequently, 50 µl of pre-diluted samples were added in duplicate to the sample wells, and 50 µl of biotin-conjugate was added to all wells, including the blanks.[23] (**Figure 3**)

The wells were then covered with adhesive film and incubated at room temperature (18-25°C) for 2 hours on a microplate shaker. Following the removal of the adhesive film, each strip underwent six washes with 400 µl wash solution. Next, 100 µl of streptavidin-HRP was added to all wells, followed by covering with adhesive film and incubating at room temperature on a microplate shaker for 1 hour. The adhesive film was removed once again, and the strips were washed six more times with 400 µl wash solution each time. Subsequently, 100 µl of TMB substrate solution was added to all wells and incubated at room temperature for 30 minutes while shielding from sunlight. Stop solution (100 µl) was added when the highest standard developed a dark blue color. Absorbance readings were taken from each microwell using a spectrophotometer at 450nm.

Statistical Analysis

Data from the study underwent manual importation into GraphPad Prism [GraphPad Software, San Diego, CA] for subsequent statistical analysis and comparison against the standard curve. The concentration values of individual growth factors, namely PDGF-BB and VEGF, were assessed. Results were presented as mean ± standard deviation derived from triplicates of each sample for every protein. Statistical analysis involved a two-way ANOVA, followed by Tukey's post hoc test to discern intergroup variations. A significance threshold of $p < 0.05$ was applied to individual sample comparisons, while $p < 0.01$ was utilized for grouped sample comparisons. Sample sizes were determined by a prior study conducted by Kim et al., wherein notable discrepancies in growth factor concentrations were observed among three patients. To enhance statistical power, this study selected 5 healthy control and 5 diabetic experimental samples for analysis.[22]

Results

L-PRF was obtained from a total of 5 healthy subjects for use as the control samples, and from 5 diabetic subjects for use as the test samples. All the L-PRF samples were centrifuged, allowed to sit for 90 minutes, and separated before being flash-frozen and stored at -80 °C. Growth factor concentrations in all ten samples were then analyzed via ELISA for PDGF-BB and VEGF at UCLA.

The standardized values of PDGF-BB were initially organized into a table of standard values (**Table 2**) and a standard curve (**Figure 4**). The data represented controlled protocol values on a logarithmic curve compared the concentrations of PDGF-BB (pg/ml) against optical density (OD). The samples were tested in duplicate twice to produce an average with standard deviation values (**Table 3**). The PDGF-BB values for each of the ten samples were represented in a bar

graph per sample (**Figure 5**) and divided based on the diabetes status whether healthy (control) or diabetic (test) (**Figure 6**). When viewing the data in Figure 5, the healthy control group showed PDGF-BB concentrations ranging from 2000 – 3400 pg/ml whereas the diabetes test group showed values ranging from approximately 1200- 4800 pg/ml. These averaged duplicate values with standard deviations depicted in Figure 6 showed similar concentrations between healthy subjects and diabetics with both averaging around 2400-2700 pg/ml. The healthy group showed an average value just shy of 3000 pg/ml with a standard deviation ranging from 2200-3500 pg/ml. The diabetes group showed an average value of approximately 2500 pg/ml with a standard deviation ranging from 1500-4000 pg/ml.

The standard VEGF values were obtained following the same procedural steps as PDGF-BB and presented as a standard curve (**Figure 7**) and a standard table of values (**Table 4**). The data for VEGF was then analyzed in duplicate and standard deviations were then calculated for each sample set and the test and control groups. All ten subjects, 5 diabetes and 5 healthy, were included in the ELISA assay and statistical analysis (**Table 5**). The VEGF values for each of the ten samples were represented in a bar graph per sample (**Figure 8**) and divided based on the diabetes status whether healthy (control) or diabetic (test) (**Figure 9**). The healthy non-diabetic control group individually displayed readings with minimal deviation. Figure 8 shows all samples, H5-H9 depicting values around 100 pg/ml. The diabetes group had greater values where D2 was near 58-76 pg/ml, D3, D4, and D6 averaged around 150-180 pg/ml with D5 having a value of approximately 230-350 pg/ml. The averages shown in Figure 9 display the test group averaging approximately 150 pg/ml while the control group average was closer to 100 pg/ml. The standard deviations in the VEGF groups generally showed narrower standard deviation intervals except those seen with D5 which ranged from 150-450 pg/ml.

Discussion

As discussed above, diabetes has numerous mechanisms by which it can hamper and impede healing and treatment outcomes. The alteration of immune cell function, encompassing neutrophils, monocytes, and macrophages, plays a pivotal role in periodontal pathology. Impairments in neutrophil adherence, chemotaxis, and phagocytosis hinder bacterial eradication within the periodontal pocket, exacerbating periodontal tissue degradation. Moreover, this dysregulation inhibits osteoblastic cell proliferation and collagen synthesis, thereby impeding bone formation and compromising the mechanical integrity of newly formed bone tissue. Fibroblast-mediated wound healing in the periodontium is also hampered due to inhibited fibroblast attachment and movement at healing sites. Furthermore, the accumulation of advanced glycation end products (AGEs) leads to the thickening of the basement membrane in the microvasculature, disrupting normal nutrient transport mechanisms and slowing tissue remodeling and turnover. Activation of the AGE binding macrophage receptor (RAGE) amplifies the production of proinflammatory cytokines like IL-1β and TNF-α, linking diabetes to periodontal inflammation.[25] This negative influence does not end here. In two different studies, Oates et al. further elaborated on implant stability among diabetic patients with high HbA1C levels. They found that the initial healing phase (2-6 weeks) might be delayed in patients with poorly controlled diabetes, resulting in a healing period twice as long as that of healthy individuals. However, at the one-year follow-up, no significant differences were observed among the three groups categorized based on their HbA1C levels: well-controlled (6.1-8%), moderately controlled (8.1-10%), and poorly controlled (≥10%).[34-35] The studies discussed above further elaborate on the multitude of ways in which diabetes negatively impacts periodontal and peri-

implant patients. These negative impacts further support the notion of exploration into the possible benefits of L-PRF as an adjunct treatment for these patients.

Delving further into this topic, other studies have investigated the relationship between diabetes and growth factor levels. A study by Shi et al. found that growth factors including fibroblast growth factor-21 (FGF21), VEGF, and TGFβ-1 were all in altered quantity, whether a deficit or an excess based on the tissue analyzed.[36] Another study looked at the effects of diabetes on growth factors including VEGF and PDGF-BB in patients being treated for diabetic foot syndrome (DFS).[37] This study found significantly higher levels of VEGF-A and PDGF-BB in patients with DFS supporting the hypothesis that subjects with diabetes will have readily higher growth factor levels in the affected tissues. In contrast, a recent study from a group in India investigated the impact of chronic inflammatory diseases on the structure and growth factor content within the PRF matrix. Using an ELISA assay, this study found that the levels of PDGF-BB were consistent across all groups.[38] The conflicting evidence highlights the need for further investigation into how diabetes will affect growth factor levels in L-PRF.

The study by Tamraz et al. investigated the influence of smoking on L-PRF, specifically how the levels of PDGF-BB, TGFβ-1, and VEGF would be affected by tobacco smoking.[11;23] The proteins chosen for analysis in this study were measurable in nanograms over time. Kim et al. similarly reported findings using L-PRF, quantifying levels of PDGF-BB, VEGF, and others at various time points for statistical comparison.[22] The protocol used in this study followed that outlined by Ehrenfast et al. where L-PRF is produced via a 12-minute centrifugation at 2700 rpm and subsequently analyzed using an ELISA assay.[15]

Platelet-derived growth Factor (PDGF) is a potent growth factor in mesenchymal cells that plays a crucial role in various physiological processes, particularly in wound healing and tissue repair.

PDGF-BB specifically refers to the dimeric isoform composed of two B chains. It is primarily secreted by platelets, as well as various other cell types including in the periodontal ligament, fibroblasts, smooth muscles, osteoblasts, and more. Upon the occurrence of tissue injury, PDGF-BB initiates various signaling cascades, leading to cellular responses such as cell proliferation, migration, and differentiation. These processes are crucial for tissue regeneration, angiogenesis, and wound healing. PDGF-BB also plays a role in regulating extracellular matrix production and remodeling. In addition to its physiological roles, PDGF-BB has been implicated in various pathological conditions, including fibrosis, atherosclerosis, and certain types of cancers. Consequently, PDGF-BB and it's signaling pathways are targets for therapeutic interventions aimed at modulating tissue repair and controlling aberrant cell proliferation in disease states.[11;39-41]

Vascular Endothelial Growth Factor (VEGF) represents a diverse glycoprotein family vital for angiogenesis, the process of generating new blood vessels from pre-existing ones. Principally produced by various cell types such as endothelial cells, platelets, and macrophages, VEGF exhibits multiple isoforms, with VEGF-A being the most extensively studied. It governs angiogenesis, vascular permeability, and wound healing by stimulating endothelial cell proliferation, migration, and survival. Anomalies in VEGF regulation are associated with conditions like cancer, diabetic retinopathy, and cardiovascular ailments. Its direct influence on endothelial cells triggers a sequence of events involving proliferation, degradation of the basement membrane, vasodilation, and chemotaxis. This vascular development significantly contributes to the creation of granulation tissue during wound healing, facilitated by platelet release post-thrombin stimulation. Additionally, VEGF engages in various interactions with

target cells including keratinocytes, fibroblasts, neutrophils, smooth muscle cells, and osteoblasts.[42-43]

The previous iterations of studies on growth factors and PRF researched how parameters such as age, gender, and time affected the material. The study at hand aimed to highlight the findings of an early exploratory pilot study depicting the differences in the concentrations of PRF growth factors between healthy non-diabetic and diabetic subjects.[22;44]

The results observed in the present study do not align with the original hypothesis of the study. The initial hypothesis was constructed with the notion that having diabetes would leave a subject in a state of chronic inflammation with increased levels of inflammatory and healing factors. The results observed by Tamraz et al. showed that patients with a history of smoking had statistically significant increases in growth factor content of L-PRF when compared to healthy subjects.[23] The observed results differ from those found in the earlier discussed studies by Shi et al. and Drela et al. Both studies found differences in the growth factor content when comparing healthy against diabetic subjects.[36-37] In contrast, the observed results do align with those found in the study from Praidou et al.; in this study, they noted that although levels of PDGF and VEGF were elevated in the vitreous samples of diabetic retinopathy patients, no such differences were noted in the samples tested from the serum.[45] Although not currently known, the mechanisms by which systemic conditions may influence growth factor content are still being investigated. One study from Nardi et al. suggests that during the progression of periodontal disease, the vasculature of the periodontium undergoes microvascular changes that result in the deterioration of supporting tissues. This suggests that diabetic patients with periodontitis exhibit notable alterations in the microvasculature of the periodontium, characterized by elevated expression of VEGF compared

to both healthy individuals and those with periodontitis but without diabetes. Recent findings support that diabetes-induced microangiopathy significantly contributes to the alteration of periodontal vasculature, thereby inducing VEGF expression through its ability to provoke microvasculopathy across various organs. However, these findings differ from earlier studies indicating elevated levels of VEGF expression in periodontal sites of systemically healthy patients compared to those without periodontitis.[46]

The observed results indicate that there may be an equal but not increased benefit of using L-PRF as an adjunct in periodontal procedures for diabetic patients compared to healthy patients. Diabetic patients are known to have impaired wound healing, especially in regenerative or grafting procedures where blood supply is pivotal.[25] The difference noted for PDGF-BB and VEGF in L-PRF of both groups was not significant and therefore adjunctive use of L-PRF may be equally beneficial for healthy and diabetes patients. Despite these findings, the data does suggest that having diabetes does not negatively influence growth factor concentrations.

Some limitations in this study may influence the overall impact. Firstly, the study had a relatively small sample size of five subjects per group from a wide age range. A larger sample size may have the ability to highlight any significant results and increase the impact of this study. Secondly, the selected cohort for the study was collected from patients of vastly different ages. When comparing the results of the growth factor concentrations against age, no definitive correlation was noted. (**Figure 10**) Despite this, some interesting trends were noted. When looking at levels of PDGF-BB in the control subjects, it is difficult to draw any conclusions due to the age distribution. (**Figure 11**) In contrast, the levels of PDGF-BB in the test group showed a trend that overall levels of PDGF-BB decreased as age increased. (**Figure 12**) When looking at

levels of VEGF in the control subjects, it is also difficult to draw any conclusions due to the age distribution. (**Figure 13**) Similar to the results with PDGF-BB, the levels of VEGF in the test group also showed a trend that overall levels decreased as age increased. (**Figure 14**) Despite the interesting trends observed, this data should be interpreted with caution as the sample size is limited, and the age ranges are not standardized across groups. These limitations further highlight the need for a study with a much larger cohort and different stratification groups. Previous data from Kim et al. and Tamraz et al. show the growth factor concentrations at much higher values than those observed in this study. One possibility for this finding could be contamination from red blood cells or acellular plasma during the isolation or preparation segments. The presence of these contents may have influenced the final readings of the ELISA assays. Some of the possible confounding variables that may have been of influence include age, gender, and other systemic conditions. Smoking for example was screened purely on history and patient reporting. This could have been an influencing factor that was not controlled. Miron et al. noted that elderly female patients have significantly larger L-PRF membranes when compared to other age and gender groups.[47] In the future, further studies investigating risk factors like smoking and diabetes or determinants (age, gender, osteoporosis, etc.) with larger sample sizes would be of great interest.

Conclusion

As an exploratory study, the present investigation was one of the first looking to specifically compare the concentration of PDGF-BB and VEGF in diabetes and healthy subjects. Within the limitations of the study, the data showed no statistical differences for both growth factors between the diabetes and healthy groups. Although not in support of the initial hypothesis, it is well documented that diabetes patients are known to have impaired wound healing, especially in

regenerative or grafting procedures where blood supply is pivotal.[25] Therefore, given this impaired status, there may still be a benefit of using L-PRF as an adjunct. Further studies are required with larger cohorts, varying HbA1c groups, and age stratification to investigate and understand the mechanisms and reasons for these observations. The findings observed from a larger-scale study would better clarify and potentially justify the adjunctive benefit of using L-PRF in patients with diabetes.

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Figures

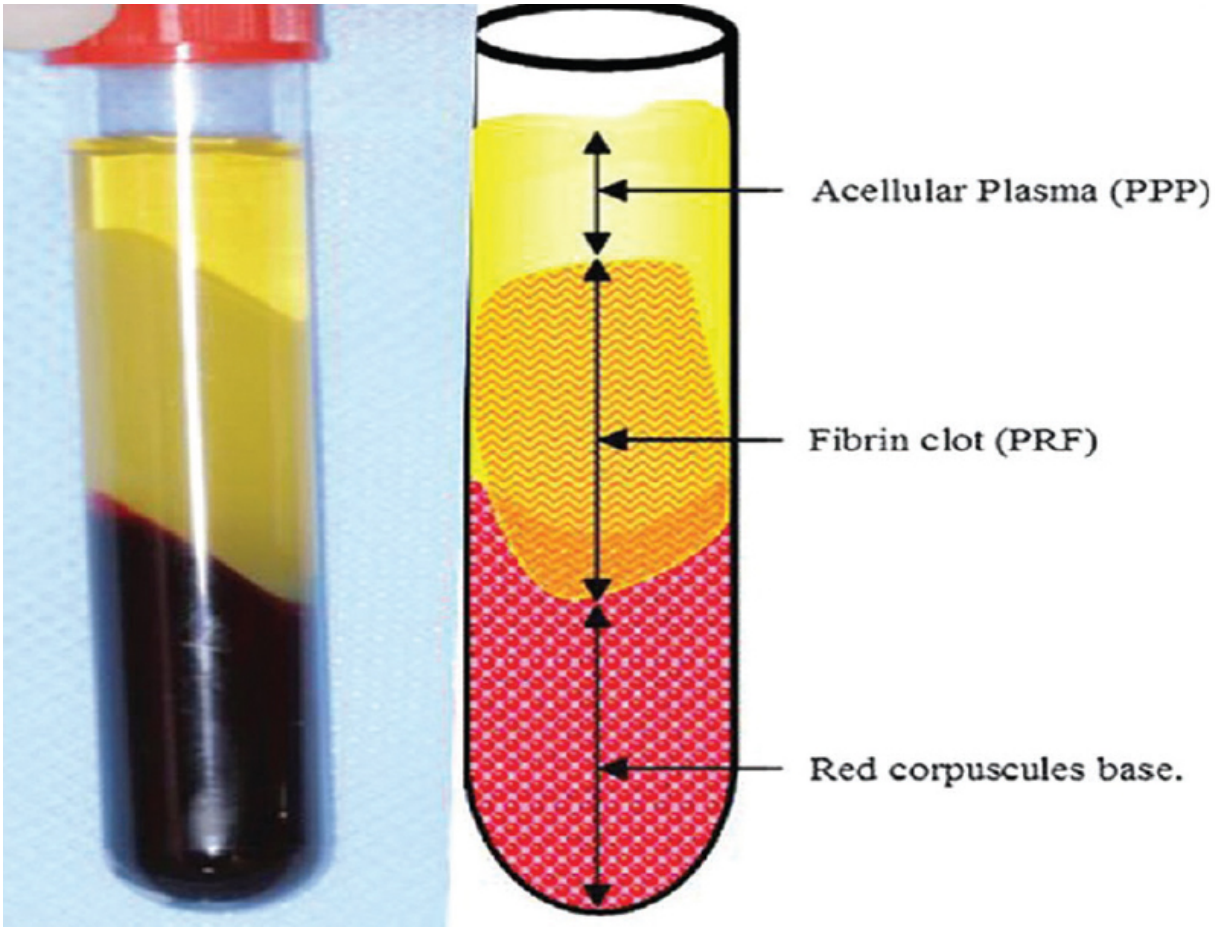

Figure 1. Sample of an Autologous Blood Draw*. The above is a diagram of a PRF sample depicting the 3 layers: an upper acellular platelet-poor plasma (PPP), the middle platelet-rich fibrin, and the red blood cells at the lowest area of the tube.*

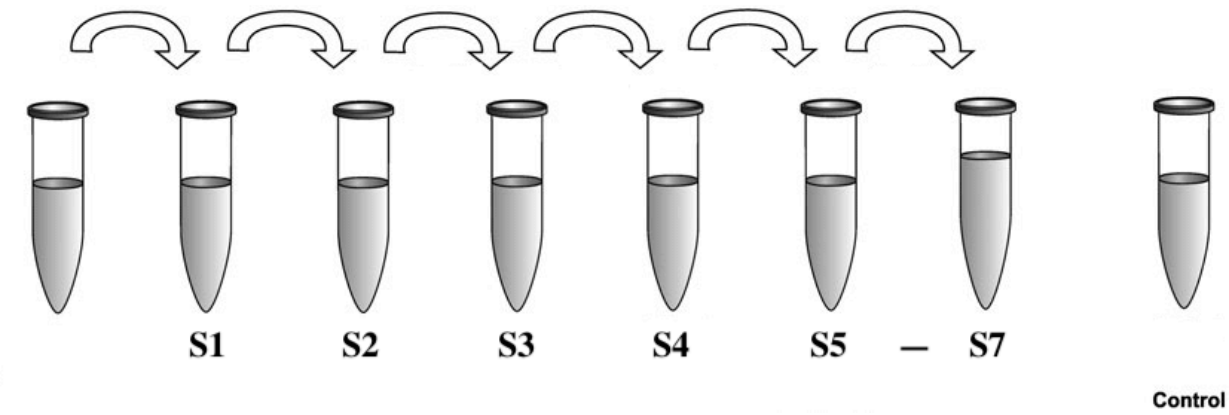

Figure 2. External Standardized Dilution. The above depicts an example of a standardized dilution with 250 µL assay buffer placed into each tube with 250µL of protein growth factor standard at S1. This was then continually diluted down to S7, with the rest discarded.

	1	2	3	4
A	Standard 1 2000 pg/mL	Standard 1 2000 pg/mL	Sample 1	Sample 1
B	Standard 2 1000 pg/mL	Standard 2 1000 pg/mL	Sample 2	Sample 2
C	Standard 3 500 pg/mL	Standard 3 500 pg/mL	Sample 3	Sample 3
D	Standard 4 250 pg/mL	Standard 4 250 pg/mL	Sample 4	Sample 4
E	Standard 5 125 pg/mL	Standard 5 125 pg/mL	Sample 5	Sample 5
F	Standard 6 62.5 pg/mL	Standard 6 62.5 pg/mL	Sample 6	Sample 6
G	Standard 7 31.3 pg/mL	Standard 7 31.3 pg/mL	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

Figure 3. Arrangement of the Samples and Standards. The above shows a sample depiction of how the standards, samples, and blanks are arranged in the ELISA microwell strips.

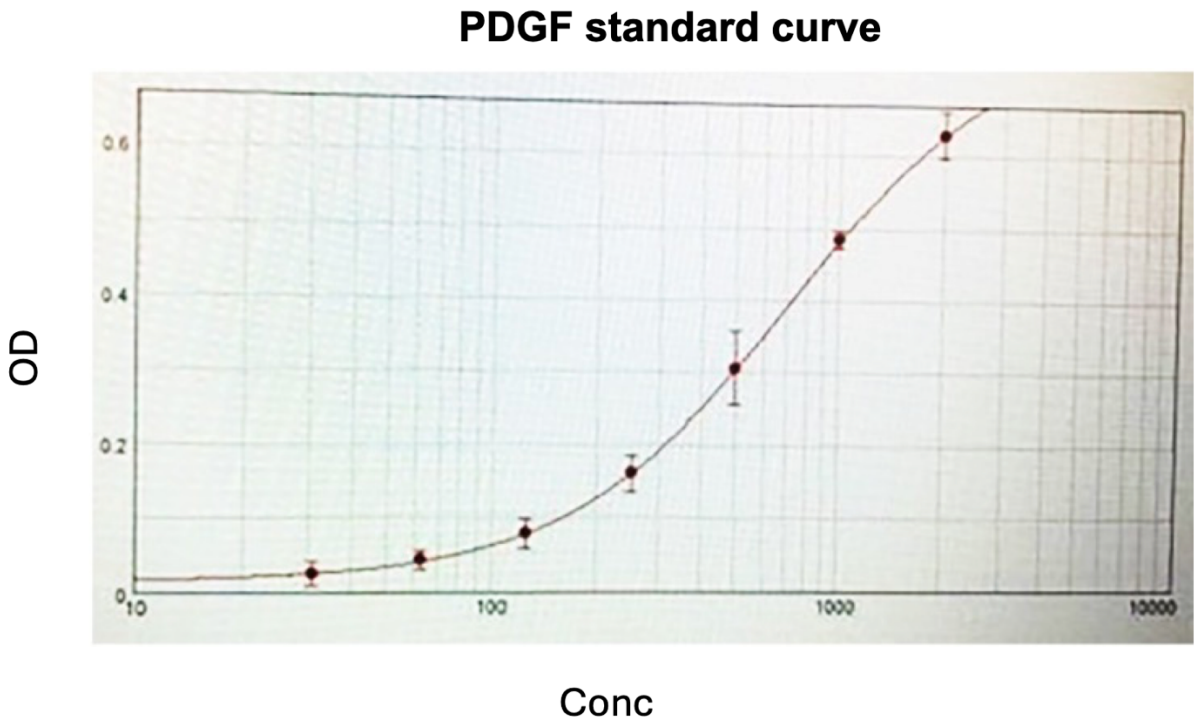

Figure 4. PDGF-BB Standard Curve. Log relationship of PDGF-BB concentration (pg/ml) to optical density (OD).

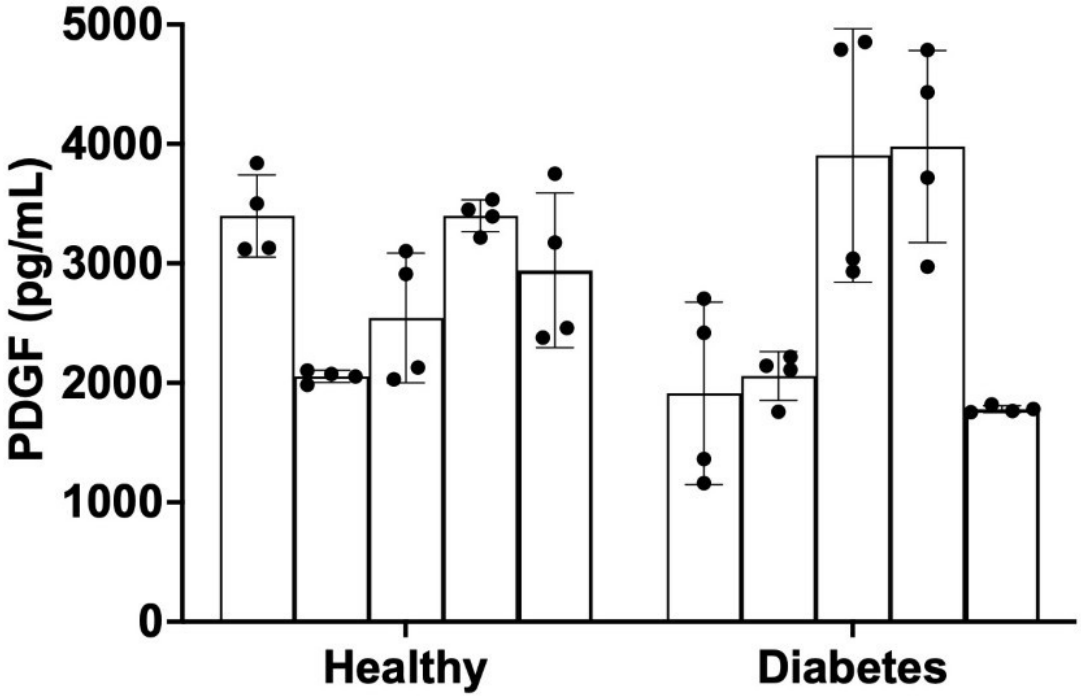

Figure 5. PDGF-BB per patient

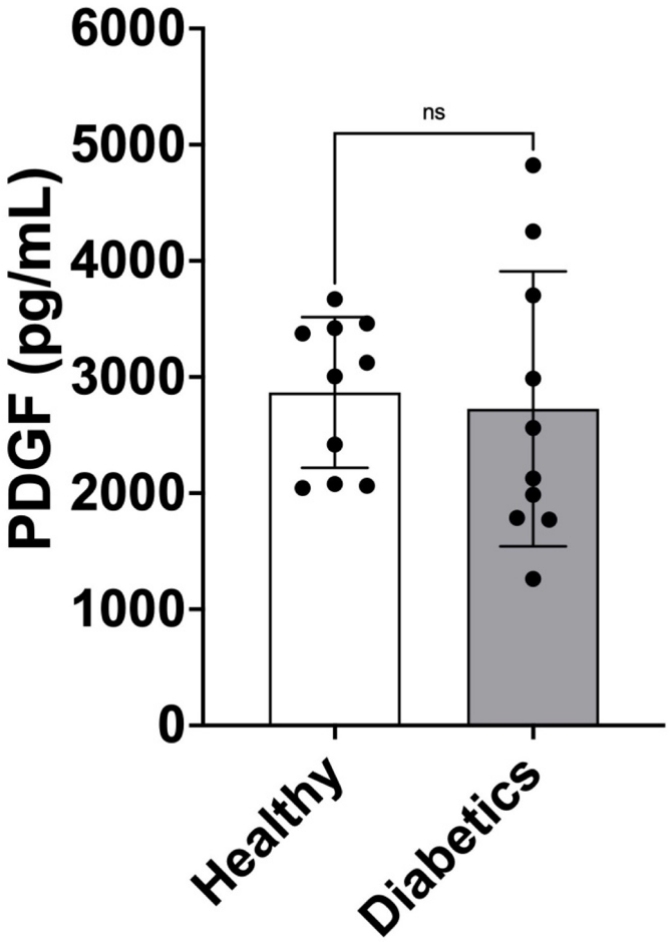

Figure 6. PDGF-grouped Healthy vs. Diabetes. ** $p < 0.01$

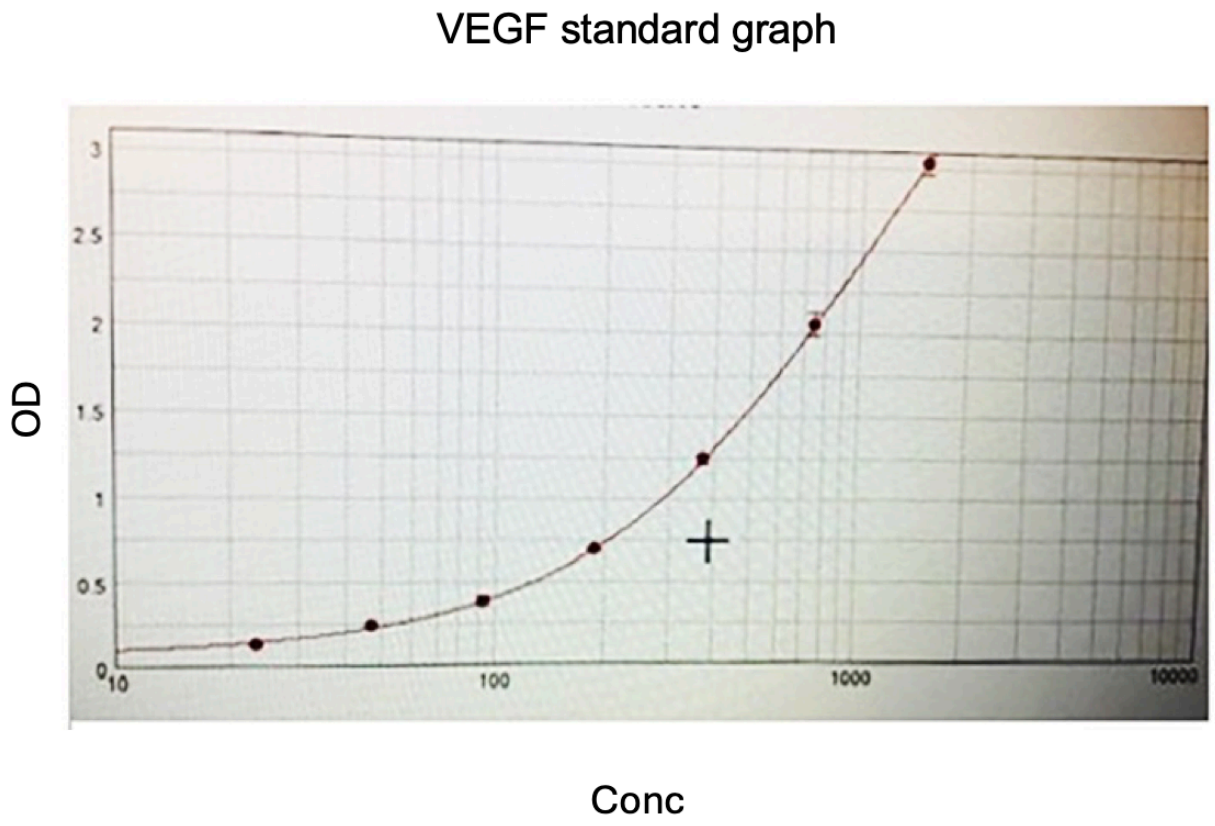

Figure 7. VEGF Standard Curve. Log relationship of VEGF concentration (pg/ml) to optical density (OD).

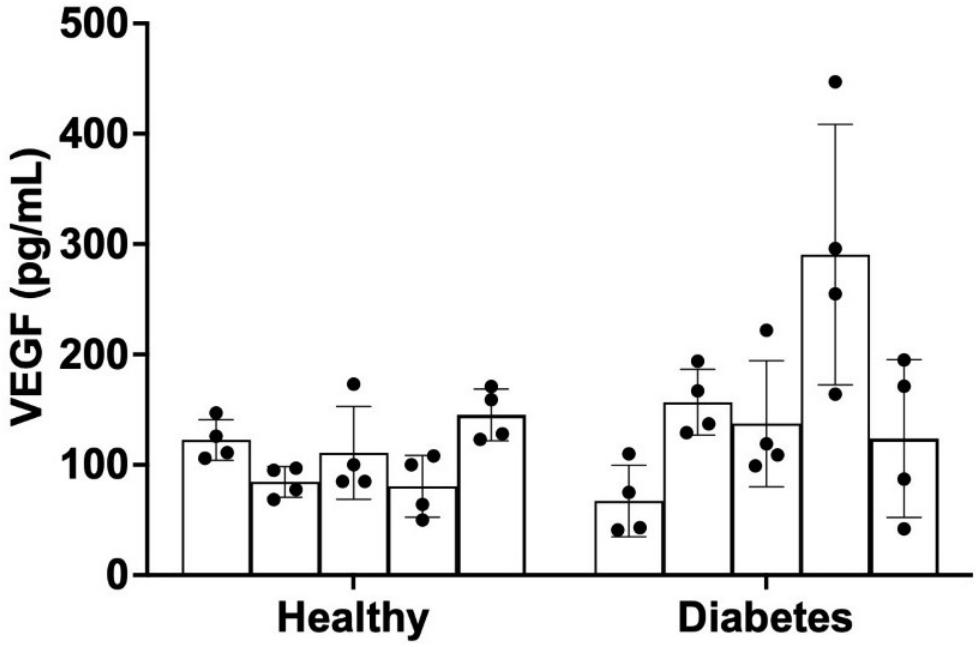

Figure 8. VEGF per patient.

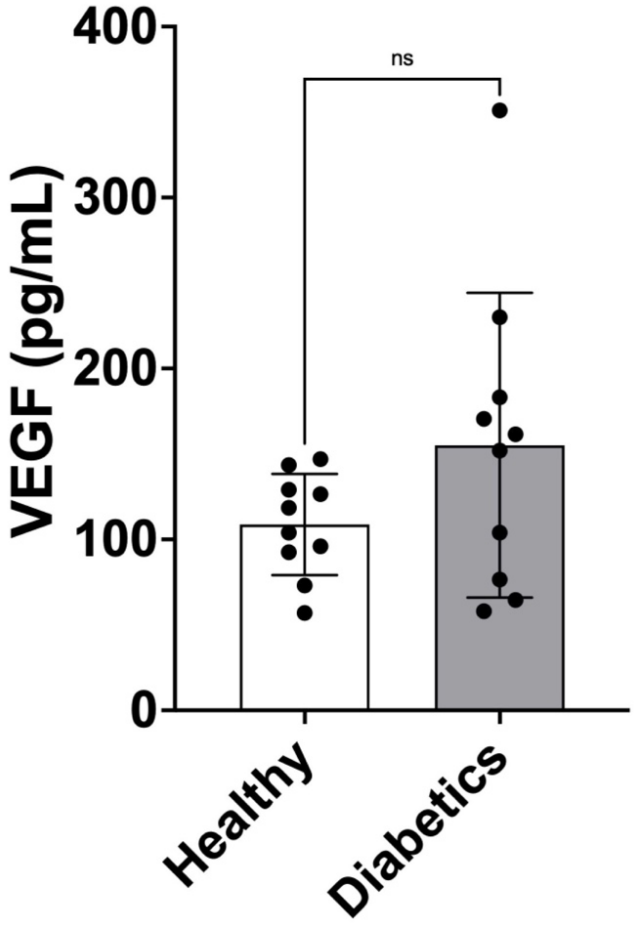

Figure 9. VEGF-grouped Healthy vs Diabetics. *p < 0.05

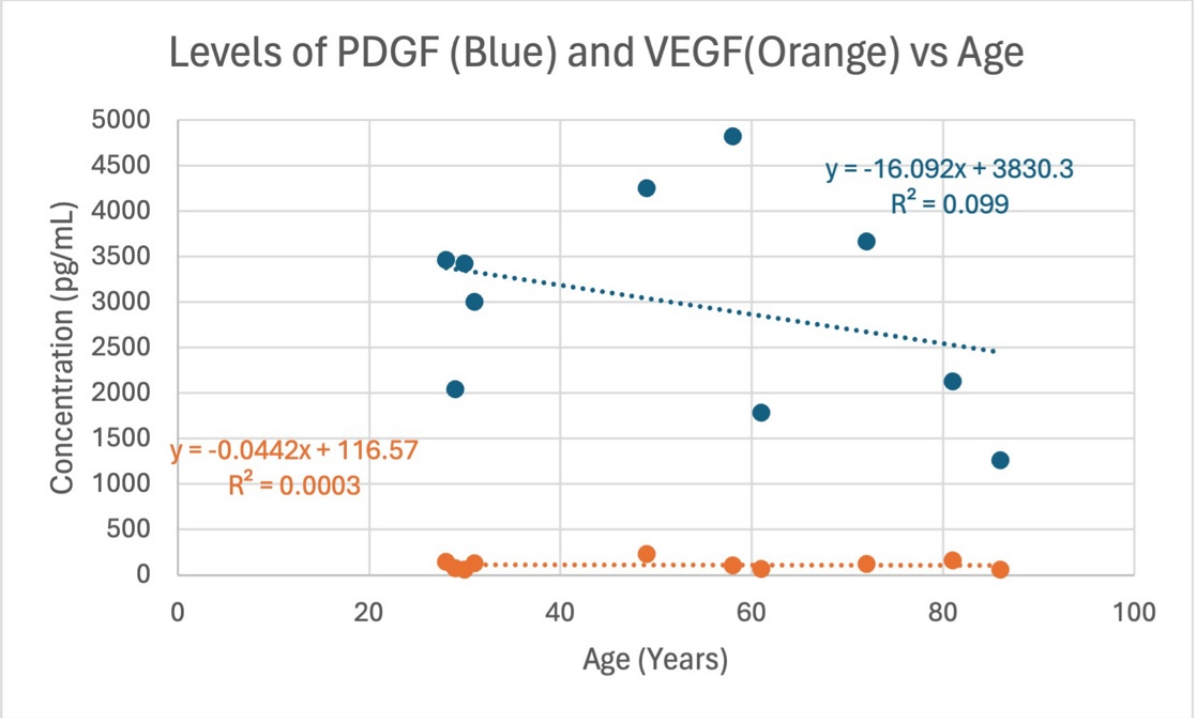

Figure 10. Levels of PDGF and VEGF versus Age (All Subjects)

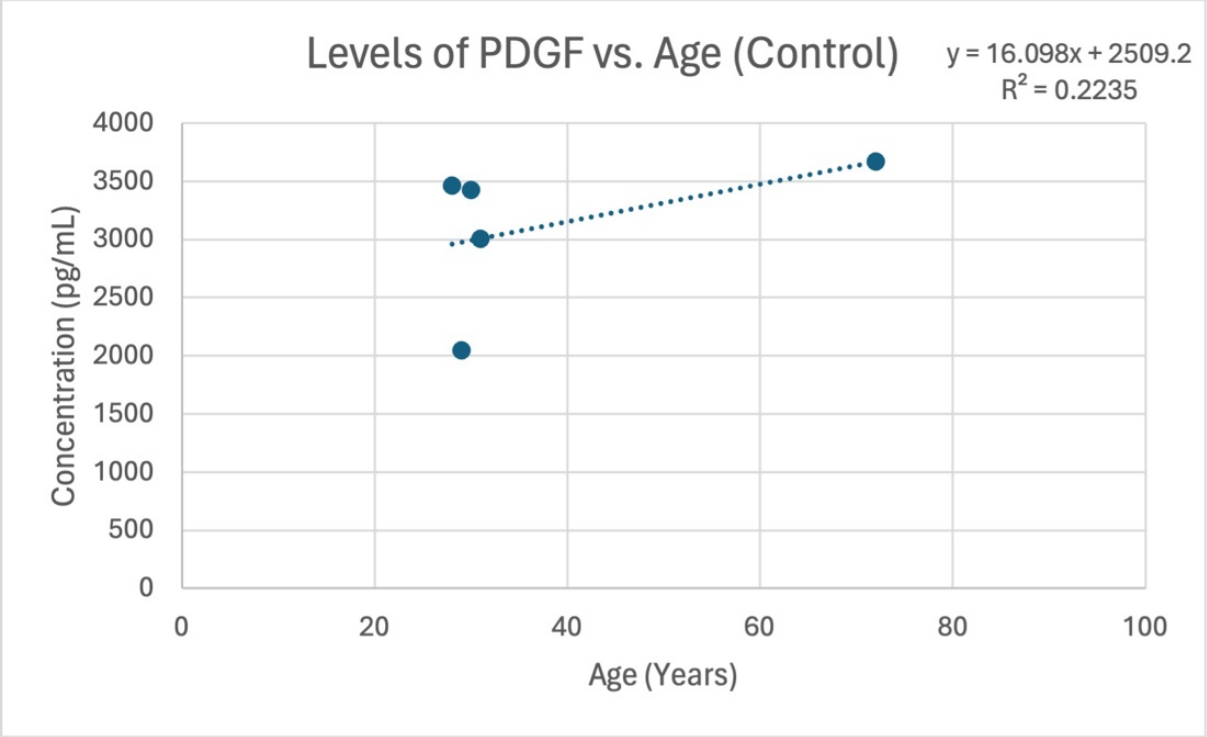

Figure 11. Levels of PDGF versus Age in Healthy Subjects

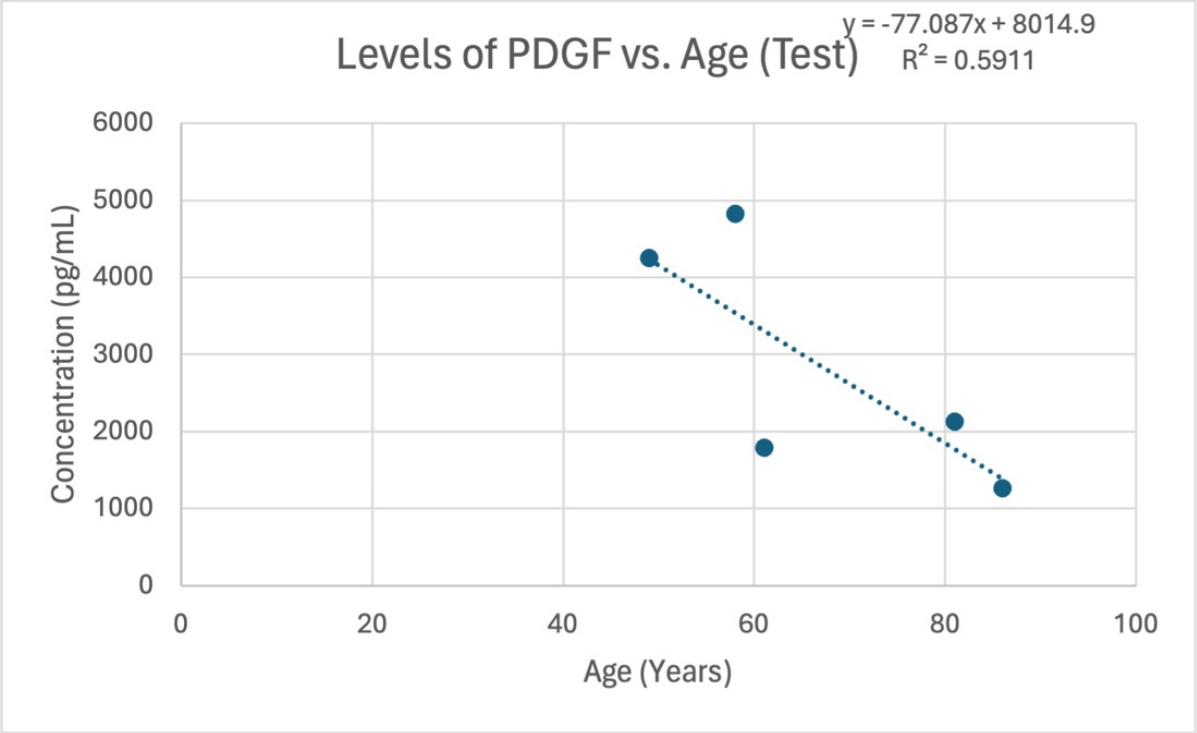

Figure 12. Levels of PDGF versus Age in Diabetic Subjects

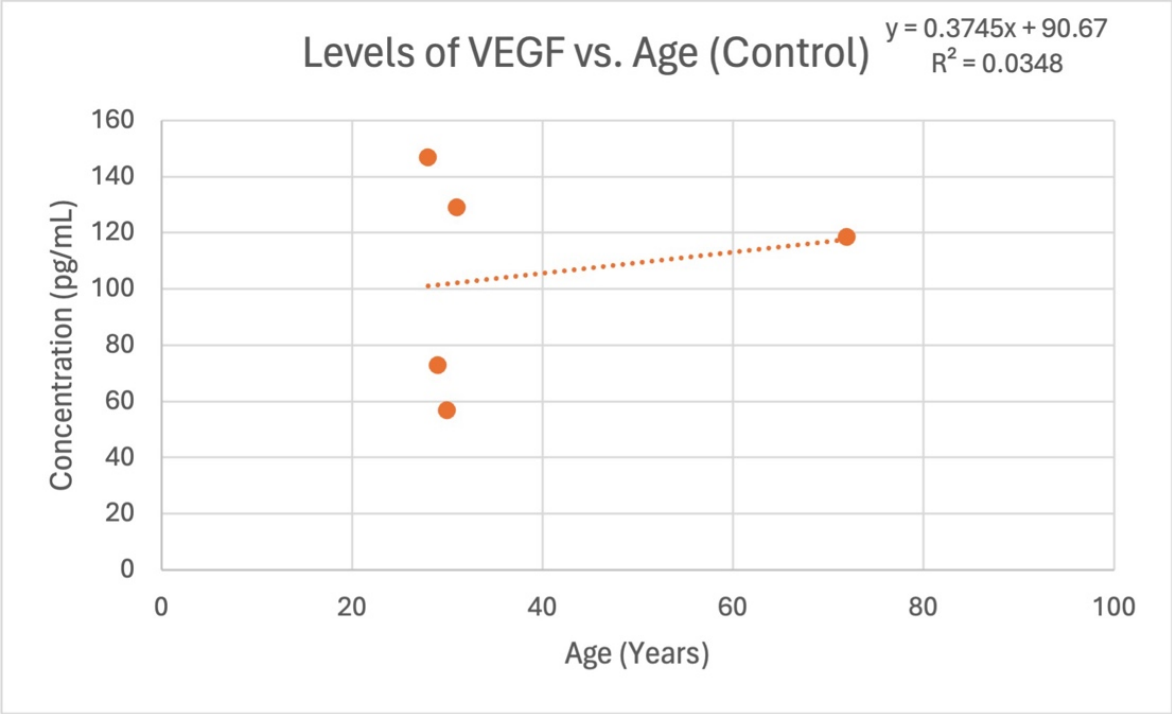

Figure 13. Levels of VEGF versus Age in Healthy Subjects

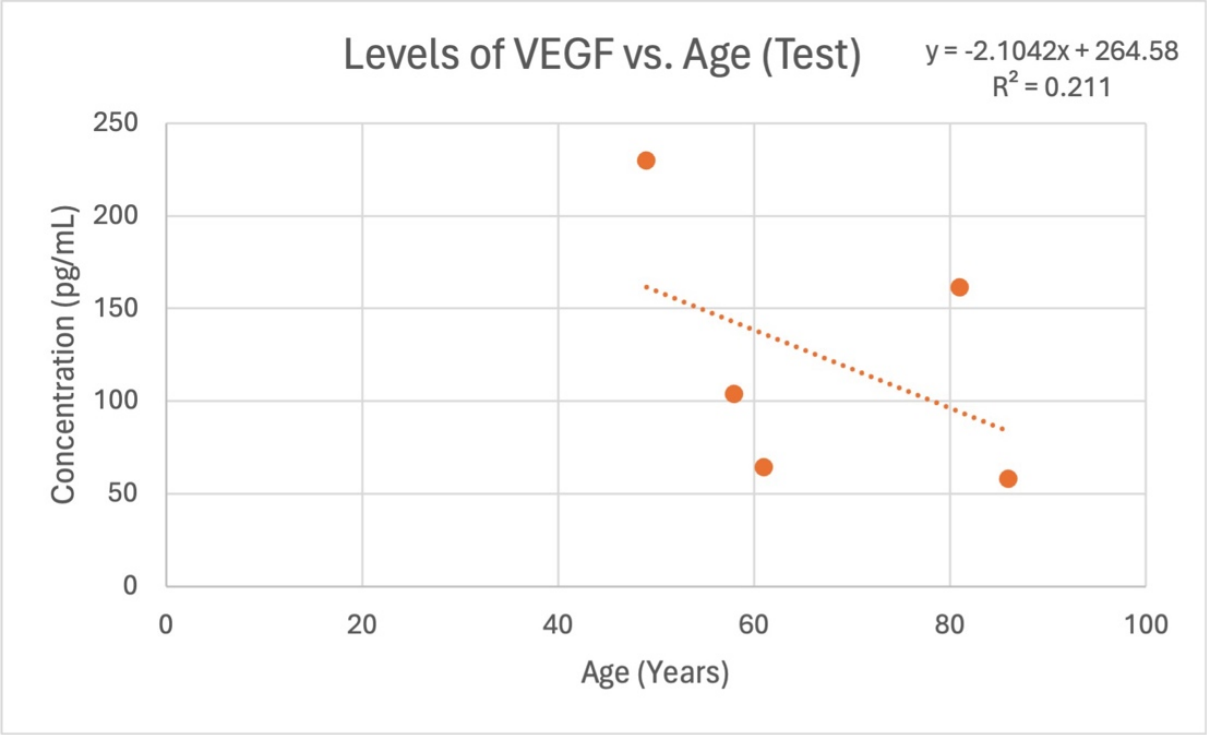

Figure 14. Levels of VEGF versus Age in Diabetic Subjects

Table 1. Sample Information.

Patient Sample Designation	Date of Blood Draw	Medical History
H5	11/01/2023	Healthy Patient - No history of smoking, diabetes, or medical conditions/medication.
H6	01/30/2024	Healthy Patient - No history of smoking, diabetes, or medical conditions/medication.
H7	01/30/2024	Healthy Patient - No history of smoking, diabetes, or medical conditions/medication.
H8	01/30/2024	Healthy Patient - No history of smoking, diabetes, or medical conditions/medication.
H9	01/30/2024	Healthy Patient - No history of smoking, diabetes, or medical conditions/medication.
D2	01/19/2024	HbA1c: 8.8%
D3	11/15/2023	HbA1c: 9.2%
D4	01/09/2024	HbA1c: 7.6%
D5	12/12/2023	HbA1c: 7.7%
D6	01/19/2024	HbA1c: 8.9%

Table 2. PDGF-BB Standard Values

Standard-PDGF	Concentration 1	Concentration 2	Average (pg/ml)
2000	2347.8	1739.4	2029.066667
1000	962.1	1036.7	999.6
500	578	431.8	503.2666667
250	275.9	222.8	249.5666667
125	144.9	102.4	124.1
62.5	81.4	49.2	64.36666667
62.5	52.4	32.5	49.13333333

Table 3. PDGF-BB Sample Concentration

4/20/2024- PDGF			
Sample	**Concentration 1**	**Concentration 2**	**Average**
H5	3130	3119	3124.5
H6	2052	2074	2063
H7	2129	2029	2079
H8	3534	3217	3375.5
H9	2460	2379	2419.5
D2	2705	2419	2562
D3	2216	1758	1987
D4	2933	3039	2986
D5	2973	4432	3702.5
D6	1764	1781	1772.5
5/8/2024-PDGF			
Sample	**Concentration 1**	**Concentration 2**	**Average**
H5	3500	3840	3670
H6	1984	2104	2044
H7	2911	3102	3006.5
H8	3393	3450	3421.5
H9	3175	3750	3462.5
D2	1363	1160	1261.5
D3	2110	2143	2126.5
D4	4791	4854	4822.5
D5	3718	4787	4252.5
D6	1820	1755	1787.5

Table 4. VEGF Standard Values

Standard-VEGF	Concentration 1	Concentration 2	Average (pg/ml)
1500	**1451.3**	**1550.3**	**1500.8**
750	723	777.2	750.1
375	369.6	383.2	376.4
188	185.3	186.3	185.8
93.8	99.3	87	93.15
46.9	52.1	51.1	51.6
23.4	21	19.2	20.1

Table 5. VEGF Sample Concentrations

4/20/2024-VEGF			
Sample	**Concentration 1**	**Concentration 2**	**Average**
H5	**106**	**147**	**126.5**
H6	97	95	96
H7	100	85	92.5
H8	108	100	104
H9	128	159	143.5
D2	43	110	76.5
D3	137	167	152
D4	119	222	170.5
D5	447	255	351
D6	171.2	195	183.1
5/8/24-VEGF			
Sample	**Concentration 1**	**Concentration 2**	**Average**
H5	**126**	**111**	**118.5**
H6	68.5	77.5	73
H7	85	173	129
H8	50	64	57
H9	123	171	147
D2	41	75	58
D3	129	194	161.5
D4	109	99	104
D5	296	164	230
D6	42	87	64.5

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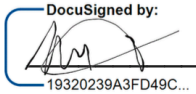

Author Signature

5/20/2024

Date