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Differences in Concentration of Growth Factor Proteins in PRF among Smokers and Non-Smokers: An Exploratory Study

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Differences in Concentration of Growth Factor Proteins in PRF among Smokers and Non-Smokers: An Exploratory Study

by

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Differences in Concentration of Growth Factor Proteins in PRF among Smokers and Non-Smokers: An Exploratory Study

Beniel Tamraz

Abstract

Platelet-rich plasma was initially introduced by Choukroun in 2000 as an autologous blood concentrate prepared through centrifugation with applications in oral surgery.[1] Today, applications of PRF include soft tissue grafting, ridge preservation, bone grafting, and sinus lift procedures, with significant benefits shown in wound healing, clinical outcomes, and material handling.[2-5] Leukocyte-PRF (L-PRF) is defined as centrifugation of autologous blood at 2700rpm for 12 minutes.[21] Investigations have shown L-PRF to release greater concentrations of specific growth factors, namely PDGF-BB, TGFβ-1, VEGF, which play a role in angiogenesis, wound closure, and immune function, and were selected for evaluation in this study.[8-11; 15; 27-32] There have been no previous studies analyzing the effects of cigarette-smoking on the content of growth factors in L-PRF. Smoking as a proven periodontal risk factor shows harmful effects on wound healing, immune function, and regenerative capabilities in periodontal surgeries.[16-20; 22-26] The aim of this case control pilot study was to compare possible differences in growth factor concentrations in L-PRF samples obtained from cigarette-smokers versus those from non-smoker healthy individuals. Three 10ml glass tubes of autologous blood were collected per patient in a pool of five healthy non-smoking patients and four current smokers. Analysis through enzyme-linked immunosorbent assays (ELISA) showed statistically significant differences (increases) in growth factor concentrations for PDGF-BB and VEGF in smokers compared to healthy non-smoker controls. TGFβ-1 levels were not in a detectable range. These results suggest a possible benefit to applying L-PRF for periodontal surgery in patients who are smokers.

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1. Introduction

Platelet-rich plasma (PRP) was initially developed by Dr. Choukroun in 2000 for development as a biomaterial in oral and maxillofacial surgery.[1] With more recent advancements, the studies on platelet-rich fibrin (PRF) have shown clinical benefits in technical handling, wound healing, and surgical outcomes.[2-5]

Platelet-rich fibrin is an autologous blood concentrate taken from the patient's intravenous blood. Through the process of centrifugation, the platelet cells aggregate, activate, and release numerous cytokines, some of which include PDGF-BB, TGFβ-1, and IGF-1.[6-8] A variety of centrifugation protocols exist, including injectable, leukocyte, and advanced PRF. Leukocyte platelet rich fibrin (L-PRF) is one protocol for platelet concentrate, specifically designated by a 2700rpm centrifugation protocol for 12 minutes.[21] Scanning electron microscopy and ELISA studies have demonstrated that L-PRF releases a greater quantity of TGFβ-1, PDGF-AB, VEGF, and BMP2 when compared to other PRF protocols.[9] Within these protocols, placement of PRF in dental surgical sites directly affected angiogenesis, increased immune function, and improves wound closure.[10-11]

Recently, autologous fibrin glue was developed as a biomaterial to enhance growth factor content in bone grafting materials. Also known as "sticky bone," the protocol includes using plastic tubing with autologous blood centrifugation in order to concentrate the factors in a viscous form.[12] This solution, mixed with allograft or xenograft hard tissue bone graft, allows for simplified hard tissue graft handling in conjunction with local cytokine and growth factor release.[13-14]

Recent studies have highlighted temporal cytokine release from the growth factors present in PRF. Specifically, IGF-1, VEGF, and PDGF-BB were found to have optimal release at 1.5 hours

after centrifugation.[15] However, the impact of a patient's systemic status on PRF growth factor concentration has not been studied; specifically, the effect of cigarette-smoking has not been evaluated.

Cigarette-smoking is especially pertinent to periodontics as it has been shown to be a risk factor for chronic periodontitis.[16-17] Mechanistically, smoking specifically decreases both innate and adaptive immunity, increases local pro-inflammatory cytokines, and decreases fibroblast attachment on tooth root surfaces.[18-20] Smoking has also been shown to play contradictory roles in terms of increasing or decreasing growth factor content in serum.[35-37]

Investigating possible differences in PRF dynamics in patients who smoke may help to understand PRF's clinical utility and potential therapeutic use. It is hypothesized that there may be minimal to no difference in growth factor protein content in smokers, leading to possible clinical benefit of using L-PRF for those with compromised periodontal surgery response. The aim of this exploratory study was to evaluate growth factor concentrations (PDGF-BB, TGFβ-1, VEGF) in PRF obtained from cigarette-smoking and non-smoking individuals.

2. Materials & Methods

2.1 Patient population and enrollment

Volunteers were enrolled from the Division of Periodontology at UCSF and were divided into two subgroups: non-smoking (healthy) and smoking. Five of the volunteers (age ranging from 29 to 71) were categorized as healthy with no reported history of diabetes (type 1 or 2) or smoking (including marijuana/recreational drugs). Four volunteers (age ranging from 43 to 70) were placed into the smoking group with reported currently smoking cigarettes both >10 cigarettes/day or <10 cigarettes/day, and with no history of diabetes (type 1 or 2). Volunteers taking blood thinners or anticoagulants were excluded. There were no incentives to participate,

and those that agreed to participate signed consent forms. Patient data was organized by blood draw date and medical history between healthy, non-smoking (H) and smoking (S). (**Table 1**)

2.2 Sample extraction

Venous blood was collected via venipuncture from the antecubital vein using two 10ml sterile glass tubes and one 10ml sterile plastic tube. The tubes were centrifuged at 2700rpm for 12 minutes using the L-PRF protocol with an Intra-Spin centrifuge [Intra-Lock International, Birmingham, AL].[21] After centrifugation, one glass test tube was labeled per patient and stored in a -80°C laboratory refrigerator after 1.5 hours.[15] The remaining two tubes were utilized for the patient's corresponding periodontal surgery on the same day. The glass tube samples were then shipped to the UCLA Section of Periodontology laboratory in biohazard-compliant dry ice containers for Invitrogen ELISA growth factor analysis.

2.3 Sample preparation

All manipulation of PRF was conducted in a biosafety level 2 plus laminar flow hood. The PRF from the tubes were semi-thawed from -80°C to nearly room temperature at 20°C to safely extract the sample from the container. In the semi-thawed state, the middle PRF layer of the sample was separated into three pieces with a sterile blade. The upper acellular plasma layers, and bottom red blood cell layers were discarded in a biosafety-compliant dispensing container. Each one third PRF sample was run through the corresponding 3 protein ELISA assay kits after placing at room temperature at 20°C. (**Figure 1**)

2.4 ELISA Analyses of Growth Factors

Invitrogen enzyme-linked immunosorbent assay (ELISA) was completed based on the Invitrogen [Thermo Fisher Scientific, Waltham, MA, USA] protocol kit instructions for the three growth factor proteins: PDGF-BB, VEGF, TGFβ-1. The reagents were initially prepared with buffer

concentrates brought to room temperature, starting with wash buffer. The 50 ml wash buffer concentrate (20x) was poured into a 1000 ml graduated cylinder and diluted to 1000 ml with deionized water and mixed. The wash buffer was stored at 2°C. Assay buffer (5 ml) was poured into a 100 ml graduated cylinder and brought to 100 ml final volume with distilled water, then stored at 2°C. Biotin-conjugate was diluted to 1:100 with assay buffer in a clean plastic tube. Streptavidin-HRP was diluted to 1:100 with assay buffer. The test protein was reconstituted with assay buffer for 15 minutes and mixed for homogenous solubilization (4000 pg/ml).

External standard dilution was thus completed with 7 tubes for standard points, labelled S1-7. 2-fold serial dilutions were prepared with pipetting 250 µl assay buffer into each tube. 250 µl of reconstituted standard (concentration 4000 pg/ml) was placed into the first tube S1 and mixed (concentration S1 = 2000 pg/ml). 250 µl of this dilution was placed into the second tube S2, and mixed. Serial dilutions were repeated 5 more times to create the standard curve points. (**Figure 2**)

Test protocol began with pre-diluting the sample to 1:10 with assay buffer, with the formula 20 µl sample and 180 µl assay buffer. Microwell strips were removed from the holder and stored in a foil-covered bag with desiccant at 2°C. Microwell strips were twice washed with 400 µl wash buffer with aspiration between washes. The strips were tapped on an absorbent pad to remove excess wash buffer. 100 µl of standard dilutions (S1-7) were pipetted into the standard wells, with 100 µl assay buffer added in duplicate to the blank wells, and 50 µl assay buffer added to sample wells. 50 µl pre-diluted samples were added in duplicate to sample wells. 50 µl biotin-conjugate was added to all wells including the blank wells. (**Figure 3**)

The wells were covered with adhesive film and incubated at room temperature (18-25°C) for 2 hours on a microplate shaker. After removing adhesive film, the strips were washed six times

with 400 µl wash solution. 100 µl streptavidin-HRP was added to all wells, then covered with adhesive film and incubated at room temperature and set on a microplate shaker for 1 hour. Adhesive film was again removed, and strips were washed six times again with 400 µl wash solution. 100 µl TMB substrate solution was added to all wells and incubated at room temperature for 30 minutes covered from sunlight. Stop solution 100 µl was added when the highest standard developed a dark blue color. Absorbance was read on each microwell on a spectrophotometer at 450nm.

2.5 Statistical analysis

Data was imported into GraphPad Prism [GraphPad Software, San Diego, CA] manually for statistical analysis and comparison to the standard curve. The concentration values for each growth factor were analyzed based on corresponding three growth factors PDGF-BB, VEGF, TGFβ-1. Data was presented as mean standard deviation from triplicates of each sample for each protein. Data was analyzed using a two-way ANOVA, and intergroup differences were analyzed by Tukey's post hoc test. Comparing groups between healthy non-smokers and smokers, $p < 0.05$ for each sample, and $p < 0.01$ for grouped samples. Sample sizes were determined by a similar previous study from Kim et al. where three patients in their study showed reportable differences in growth factor concentration comparisons.[15] In order to improve the power in this study, five healthy control and four smoker experimental samples were selected.

3. Results

PRF was obtained for a total of five healthy control samples, and four smokers test samples. PDGF-BB, VEGF, TGFβ-1 were tested after 1.5 hours post-centrifugation prior to freeze -80°C and shipment to the UCLA lab for ELISA testing after thawing. Healthy non-smoker control

sample H3 was excluded from the study due to non-testable membrane volume. After excluding this sample, a total of 8 samples were evaluated for each growth factor.

PDGF-BB standards were initially collected in both a standard curve (**Figure 4**) and standard values (**Table 2**). These served the purpose of depicting the controlled protocol data on a logarithmic curve for relation of PDGF concentration (pg/ml) to optical density (OD). The samples for PDGF-BB were analyzed in triplicate to create an average with standard deviation. H4 was removed from this section due to one of the triplicate values falling out of the standard deviation, deeming this sample as an outlier (**Table 3**). PDGF concentrations for each sample were placed in a bar graph per sample (**Figure 5**) and grouped together based on test and control groups (**Figure 6**). Individual values of PDGF per patient in figure 5 show general consistency between healthy non-smoking control patients, with concentrations ranging from 2,000 to 4,000 pg/ml. Test smokers displayed a greater variety, with S1 showing comparable levels to healthy non-smoking controls. S2 to S4, however, ranged from 4,000 to 10,000 pg/ml. These triplicate averages with standard deviation, when grouped together in in figure 6 between test and control show nearly triple concentration of PDGF in smokers with average 6,000 pg/ml, with a much larger deviation from 3,000 to 8,000 pg/ml. Healthy non-smoking controls on average were near 3,000 pg/ml with a small deviation of 2,000 to 3,500 pg/ml.

VEGF values were collected in a similar manner to PDGF with a standard curve (**Figure 7**) and standard values (**Table 4**). The samples for VEGF were also analyzed in triplicate and calculated to reveal the average with standard deviation. All H1-H4 healthy non-smoking controls and S1-S4 test smokers were included in these readings (**Table 5**). VEGF concentrations were placed in a bar graph per sample, depicting the averages (**Figure 8**) and grouped together to compare average test and control (**Figure 9**). The healthy non-smoking control group in the individual

reading displayed a large range between patients with minimal deviation. Figure 8 shows H1 and H4 samples in the 500 pg/ml VEGF range, while H2 and H3 were near 1,000 pg/ml. Test smokers had greater values where S1 was near 250 pg/ml, S2 to S4 averaged around 1,500 pg/ml with deviation maximum value at 2,000 pg/ml. With the averages placed in Figure 9, the test smokers group averaged double that of the healthy non-smoking control group at 1,200 pg/ml versus 600 pg/ml. Deviation of the smoker group ranged from 500 pg/ml up to 2,000 pg/ml, while the healthy non-smoking group ranged from 500 pg/ml to 1,000 pg/ml.

The third tested protein TGFβ-1 was placed into standard value (**Table 6**) and standard curve (**Figure 10**). All samples in the study H1-H4 and S1-S4 were placed in the test. However, when scanned, the TGF sample data was not in the detectable range with the corresponding optical density (**Table 7**). Conclusions based on the TGF protein analysis were therefore excluded.

4. Discussion

This study evaluates the differences in concentration of three growth factors in L-PRF between cigarette-smokers and healthy patients. By assessing these differences, it may be possible to discern the validity of using PRF in patients with compromised wound healing and immune function undergoing dental surgery. Although this is a relatively limited exploratory study, it may lead to further ongoing research with larger sample size and other systemic conditions, such as diabetes. For this reason, any history or current diabetes status (both type 1 and type 2 diabetes) was excluded from this study as a possible confounding factor. Consideration for type 1 or type 2 diabetes systemic effect on PRF growth factor concentration would be beneficial in a separate study.

Cigarette-smoking is a significant concern when it comes to periodontal treatment. Namely, it has been confirmed as a risk factor for periodontitis and peri-implantitis through numerous

longitudinal randomized controlled trial studies.[23-24; 26] Recently, Leite et al. analyzed the incidence and progression of periodontitis associated with cigarette-smoking; this meta-analysis of 28 studies found that smoking increases the risk of periodontitis at a mean of 85%.[22] Cigarette smoking also plays a major role in periodontal disease progression classification, where patients who smoke ≥10 cigarettes per day are classified as Grade C periodontitis.[25]

As previously mentioned, the mechanisms of smoking both affect ongoing disease and treatment. Qiu et al. study mentions a "dual role" in both heightening pathogenic immune responses and depleting defensive immunity in both innate and adaptive systems. Innate immune cells that are affected include macrophages and natural killer cells, and adaptive immune cells include T helper cells, CD8+ cells, B cells.[18] Smoking has also shown to play a role in proinflammatory cytokines; gingival crevicular fluid samples have shown to increase IL-1β levels. IL-8, a functional interleukin driving chemotaxis, is significantly decreased in smokers.[19] PDL fibroblasts are also found to deplete function in attachment to root planed surfaces. In such, periodontal regeneration after treatment is severely compromised.[20] Such evidence verifies the multi-mechanistic nature of smoking that involves decreased wound healing, immune function, and local regenerative capabilities.

Previous studies have shown various patterns of risk association in affected growth factors in smokers compared to healthy patients. One such study from Erlandsson's group investigated the effect of smoking in rheumatoid arthritis patients, specifically seeking effects on insulin-like growth factor-1 (IGF-1). ELISA tests for IGF-1, adiponectin, leptin, resistin showed statistically significant decrease in all factors when tested from serum.[35] Interestingly, another study demonstrated increased content of VEGF, PDGF, and fibroblast growth factor (FGF) in nicotine samples. This analysis consisted of human endothelial cells extracted from lungs and analyzed

through assay kits, suggesting nicotine's pleiotropic effect, and inducing angiogenesis in lung cells.[36] On the other hand, cigarette smoke extract has shown to decrease VEGF in well-differentiated lung endothelial cells. Cultures in RT-PCR and ELISA showed an inhibitory effect of the smoking extract on VEGF when induced by *Mycoplasma pneumoniae*.[37] Various patterns of growth factors and smoking apply to different biologic systems, where platelet rich samples have yet to be thoroughly investigated.

The growth factors selected for this experiment stem directly from previous studies that utilized similar clinical evaluation.[8] Ehrenfest et al. investigated the impact of centrifugation characteristics and confirmed assay findings using L-PRF (2700rpm, 12 minutes) with ELISA kits. The proteins analyzed included transforming growth factor β-1 (TGFβ-1), platelet growth derived factor-AB (PDGF-AB), vascular endothelial growth factor (VEGF). These proteins selected in this study were quantifiable in nanograms over time.[9] Kim et al. also demonstrated similar results by using L-PRF and evaluating PDGF-BB and VEGF in concentrations that were measurable for statistical purposes in comparisons.[15] This study, therefore, specifically analyzed quantities of TGFβ-1, PDGF-BB, and VEGF.

Platelet-derived growth factor-BB (PDGF-BB) is a common factor found in mesenchymal cells of the periodontal ligament, fibroblasts, smooth muscle cells, chondrocytes, and osteoblasts. Stimulation during injury results in proliferation of cells and increased stem cell markers. Additionally, collagen chains maturation is increased through lysyl oxidase (LOX) activity and secreted protein acidic and cysteine rich (SPARC) expression. Expression on endothelial cells has also shown angiogenesis activity with interactions between PDGFR-β and PDGF-B chain, along with blood vessel granulation. Secretion also stimulates collagen and bone matrix formation with bone morphogenic proteins (BMP), a potent osteoinductive factor.[8; 27-28]

TGFβ-1 is another major protein growth factor found in PRF that drives the cell growth, immunologic, and angiogenic process of wound healing. The multifunctional role begins as a latent precursor where numerous receptors retain autocrine and paracrine roles upon neighboring cells. Two of these receptors within the superfamily regulate signal transduction in both cell differentiation and maturation. Additionally, TGFβ-1 is released by T cells and B cells, where direct downregulation results in decreased interleukin-1 and interleukin-2 when over-expressed. Lastly, TGFβ-1 works in direct conjunction with VEGF in angiogenesis, where VEGF-mediated apoptosis results in TGFβ-1 induced angiogenesis on endothelial cells.[29-31]

VEGF was the third protein that was considered in this study. VEGF is formed by platelets, macrophages, endothelial cells, and functions generally include roles in chemotaxis, vascular permeability induction, and endothelial cell mitogen function. It is a homodimeric glycoprotein with similar homology to PDGF with multiple isoforms. The third tyrosine kinase receptor marks VEGF with notable roles in lymphangiogenesis. The direct role on endothelial cells results in a cascade of proliferation, basement membrane degradation, vasodilation, and chemotaxis. This blood vessel formation directly contributes to granulation tissue formation in wound healing, along with release from platelets after thrombin stimulation. Other numerous interactions include target cells in keratinocytes, fibroblasts, neutrophils, smooth muscle, and osteoblasts.[32]

This investigation illustrates an early exploratory pilot study that depicts the differences in concentration of PRF growth factors between cigarette-smokers and non-smoking healthy patients. Because most previous studies on growth factors in PRF studied kinetics, effects of age and gender, and timing, no studies have shown how periodontal risk factors affect this biomaterial.[15, 34]

The results of this study interestingly contradict the initial hypothesis of the study. Rather than cigarette-smoking negatively affecting growth factor concentration, it showed statistically significant increase in PDGF ($p < 0.01$) and VEGF ($p < 0.05$) when compared to healthy patients. TGFβ-1, however, could not be confirmed with this pattern due to sample values not in detectable range. This agrees with the study from Ebrahimpour that have depicted increased VEGF, PDGF, FGF in lung samples exposed to smoking. Mechanisms in this study provided insight into the nicotinic acetylcholine (nACh) machinery; interaction between nicotine with acetylcholine and homomeric α7 nAChR promote inflammation and fibrosis. VEGF, FGF, PDGF are thus released through cationic Ca^{2+} permeability. This leads to increased cell proliferation, migration, differentiation, and angiogenesis.[36] Other studies investigating VEGF expression in mRNA levels due to nicotine point to progression of vascular disease and cancer.[38] Though the current study does not investigate the mechanism of this increase of growth factors, suggestions may be attributed to the nicotine acetylcholine pathway.

Another possible mechanism may be linked to the presence of oral bacteria in smokers, and the effect of growth factor release. The 1996 study by Zambon demonstrated that cigarette smoking is directly correlated with increased pathogenic oral bacteria. Through indirect immunofluorescence microscopy, the investigation found increased levels of *Bacteroides forsythus*, *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* due to impaired host immune function and decreased serum IgG2.[40] These gram-negative bacteria consist of lipopolysaccharide in their membranes that act as toxins. Interestingly, Cesta's study found that bacteria with lipopolysaccharides exacerbated PDGF release in rat lung fibrosis.[41] Kim's study also found similar findings in increased expression of VEGF mRNA in response to lipopolysaccharide from rat lung models.[42] Such findings suggest it may be possible that

cigarette-smoking plays an indirect role in increasing growth factor proteins through oral pathogenic bacteria.

These results may imply some clinical benefits for using PRF in smoker patients undergoing periodontal surgery. Typically, patients who smoke cigarettes have compromised healing in periodontal procedures, namely regenerative and grafting due to vasoconstricted blood supply.[43] The statistically significant presence of growth factors VEGF and PDGF-BB in smokers PRF may justify clinical use of the biomaterial to induce improved wound healing, immune function, and angiogenesis. However, this study did not investigate clinical outcomes, and further research would be necessary to confirm this.

Limitations of this study include sample size. A greater sample size would increase the power of the study and improve significance. Additionally, the cohort in the study only included current smokers, where it may be beneficial to study past smokers as well. Previous National Health and Nutrition Examination Survey have examined the odds ratio in relation of past smoking to periodontal disease; according to this, cessation of 11 years from smoking decreases periodontitis odds ratio to 1.15.[33] This signifies residual effects of smoking, where future studies can examine this in PRF growth factors. Laboratory techniques involving immediate analysis after 1.5 hours post-centrifugation would rule out any possibility of dry ice freezing at -80°C playing a role in deviation. Additionally, a previous study from Miron demonstrated that lymphocytes, leukocytes, and platelets are greatly concentrated in the inferior portion of the PRF near the red blood cell layers.[38] Protein samples taken from different portions of the PRF may thus show variable growth factor content. Confounding factors may include the possible age range, where concentrations may vary based on age, and gender as well. This may be evident from previous studies showing less hematocrit red blood cells, and larger PRF membranes in

females and older patients.[34] Future directions for studies may investigate other forms of smoking including marijuana or vaping. Additionally, the effect of diabetes as a periodontal risk factor may also show growth factor differences in PRF.

5. Conclusion

This study was the first case control pilot study to investigate the concentration of growth factors in smokers compared to healthy non-smoking patients. Within the limitations, the data showed statistically significant increase of VEGF and PDGF-BB in the smoking group. The mechanism was not analyzed, though this may be attributable to direct results of nicotine on acetylcholine receptors, or indirect effects on gram-negative oral bacteria. Further research is required with larger sample size to confirm these findings. Additionally, analyses studying various systemic factors including marijuana use, vaping, diabetes, and past smoking history may contribute deeper insight into PRF growth factor variability. Such findings would justify clinical use of PRF in compromised patients undergoing periodontal surgery.

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Figures

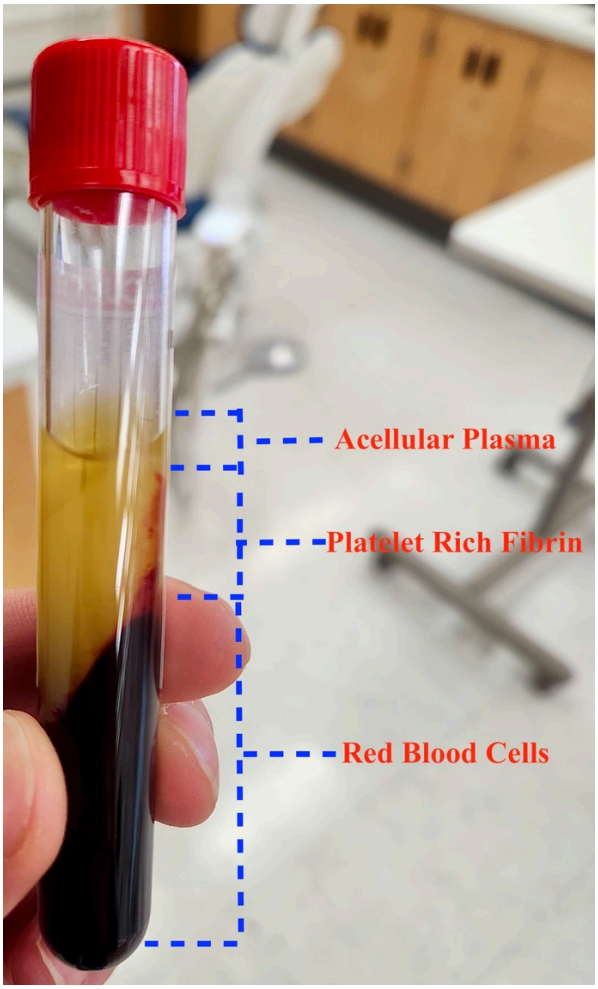

Figure 1. Autologous Blood Draw Sample. Example of a study sample displaying three layers: upper acellular platelet-poor plasma (PPP), middle later platelet rich fibrin, lower red blood cells layer.

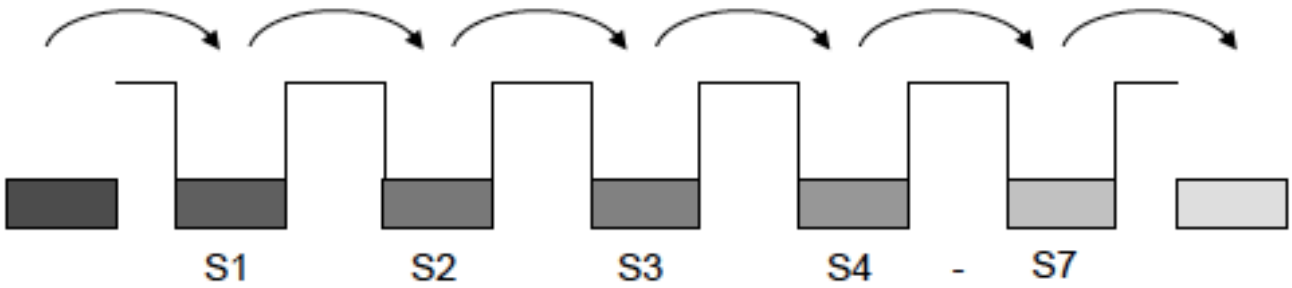

Figure 2. External Standard Dilution. Example of the standard dilution with 250 µl assay buffer placed in each tube, and protein growth factor standard was placed in S1 at 250 µl, then continually diluted to S7 with the remaining discarded.

	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. Standard and Sample Arrangements. Example of the arrangement of the standard, samples, and blank wells in microwell strips.

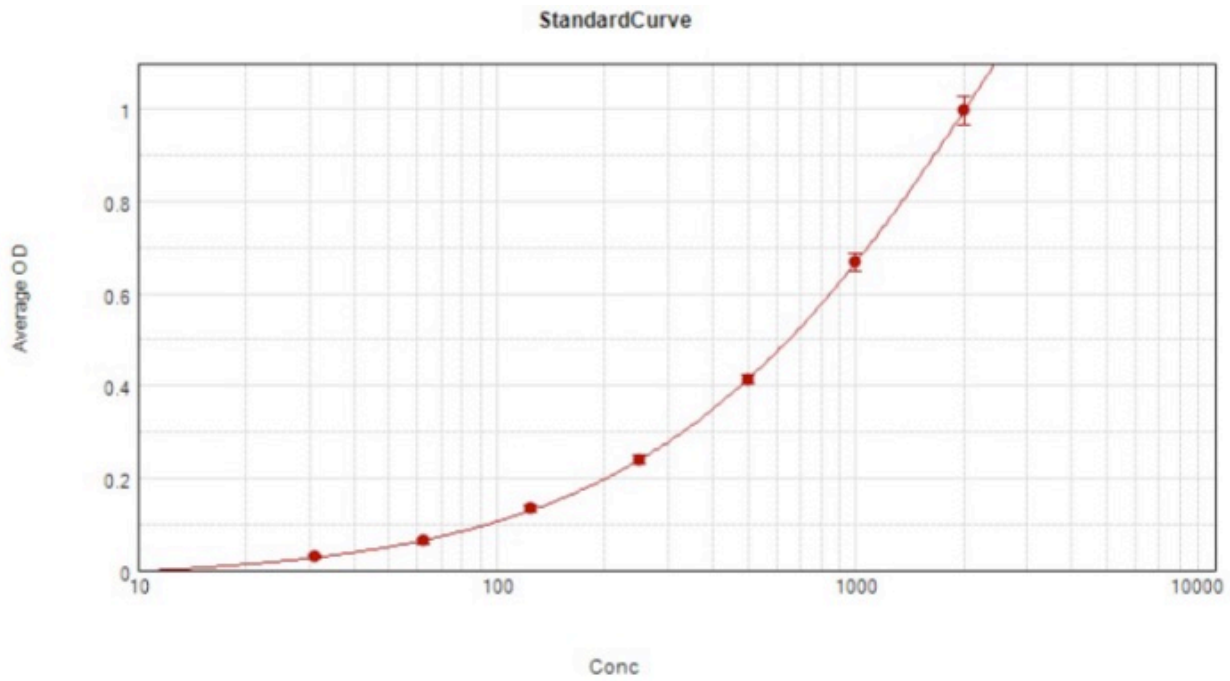

Figure 4. PDGF-BB Standard Curve. Log relationship of PDGF-BB concentration (pg/ml) to optical density (OD).

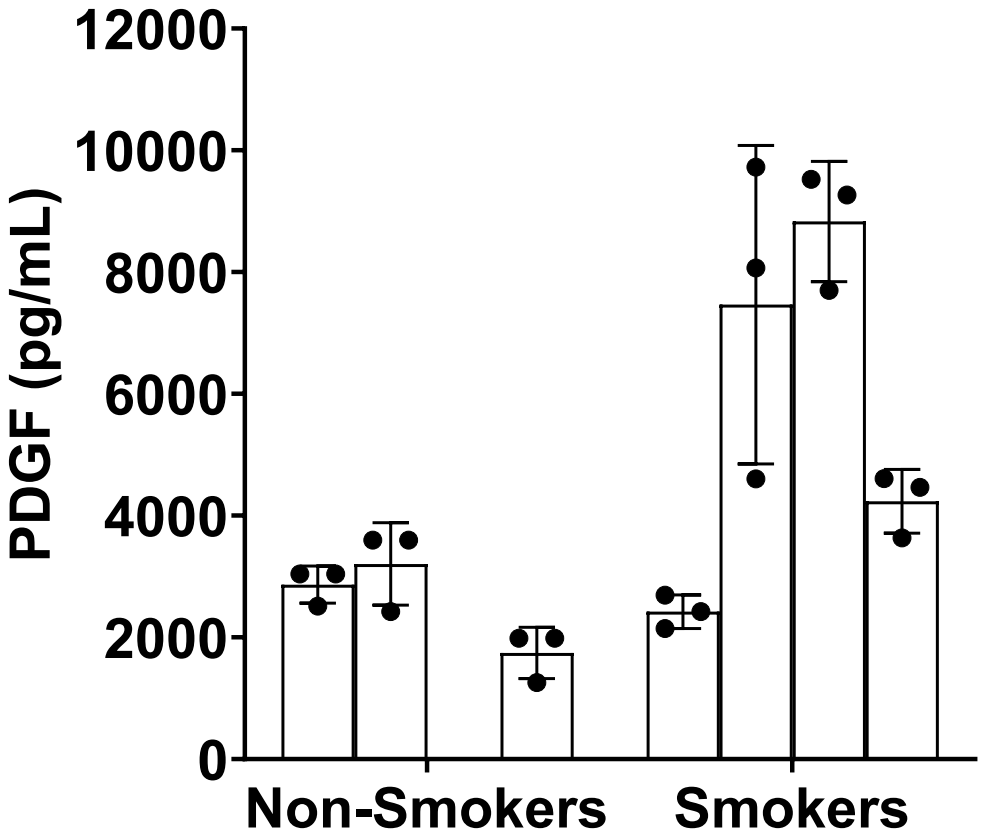

Figure 5. PDGF-BB per patient.

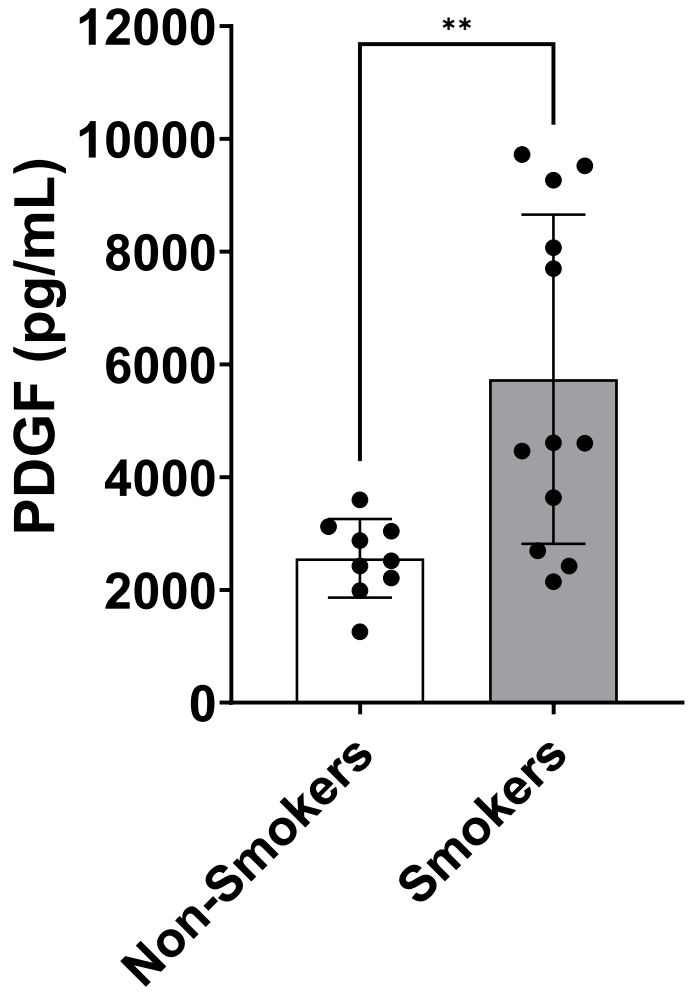

Figure 6. PDGF-grouped Healthy vs Smokers. $^{**}p < 0.01$

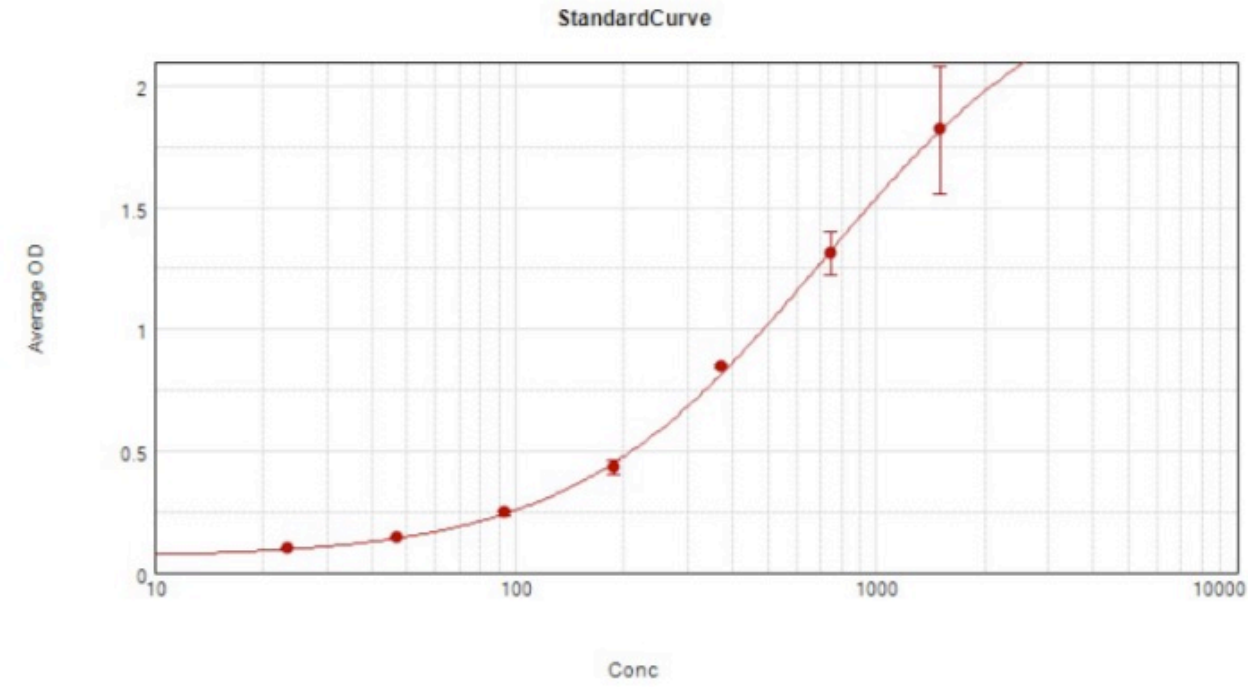

Figure 7. VEGF Standard Curve. Log relationship of VEGF concentration (pg/ml) to optical density (OD).

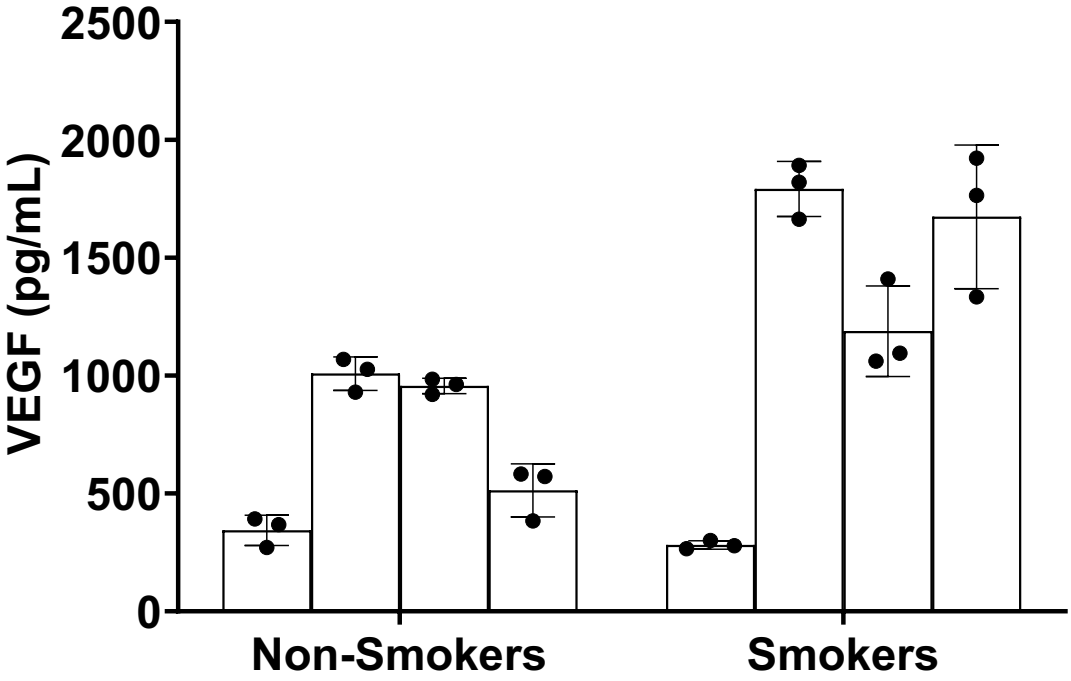

Figure 8. VEGF per patient.

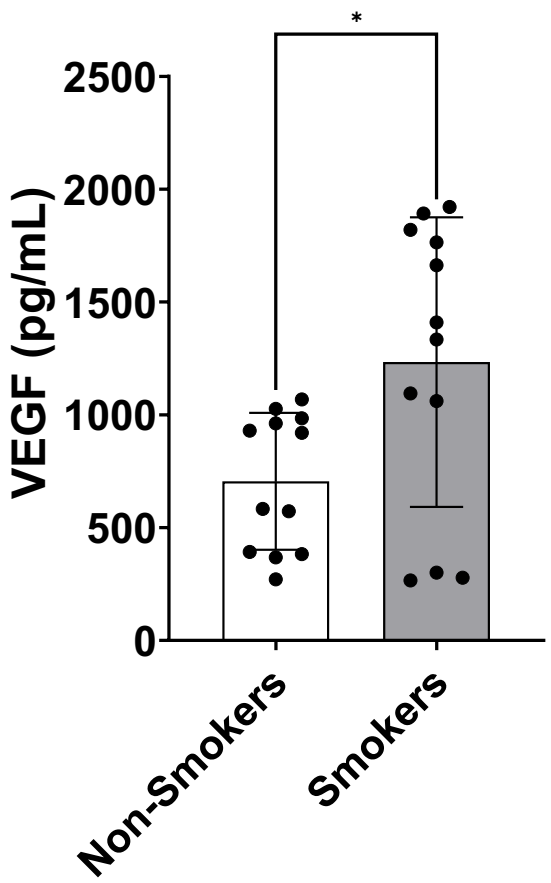

***Figure 9. VEGF-grouped Healthy vs Smokers**. *$p < 0.05$*

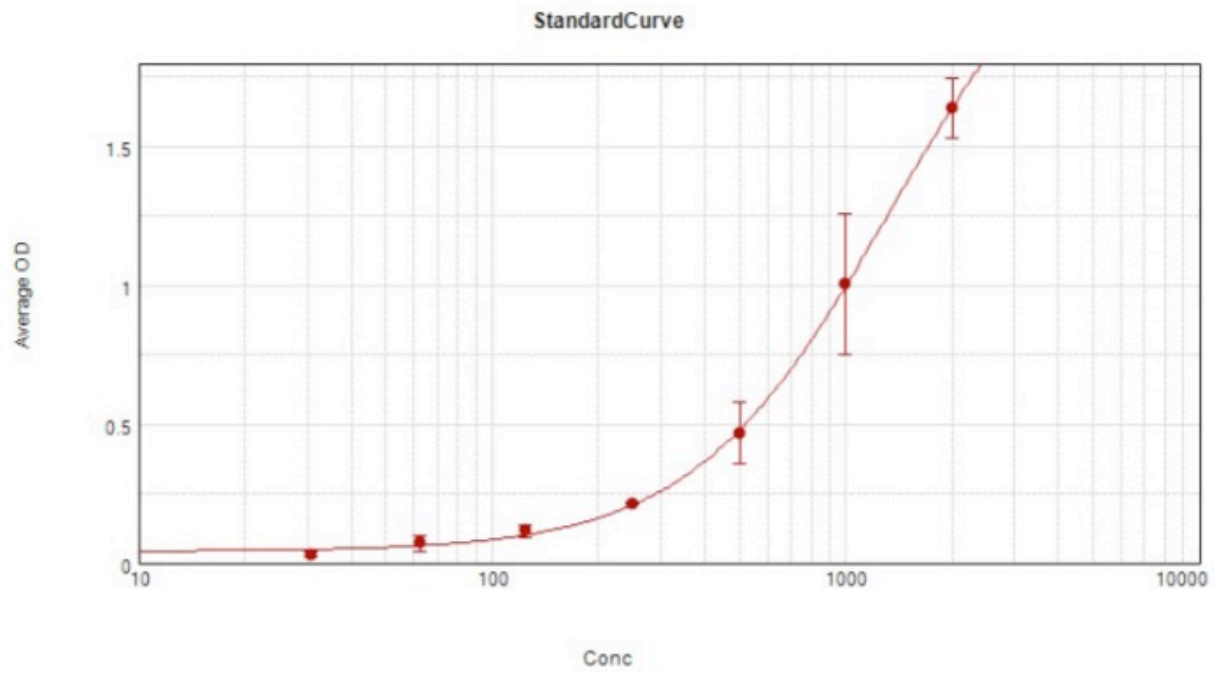

Figure 10. TGF Standard Curve. Log relationship of TGF concentration (pg/ml) to optical density (OD).

Tables

Table 1. Sample Information. **Indicates excluded from results.*

Patient Code	Date of Blood Draw	Medical History
H1	10/12/2022	Healthy patient - no smoking/diabetes/med conditions/medications
H2	10/13/22	Healthy patient - no smoking/diabetes/med conditions/medications
***H3**	10/13/22	Healthy patient - no smoking in last 40 years/diabetes/med conditions/medications
H4	1/13/2023	Healthy patient - no smoking/diabetes/med conditions/medications
H5	11/1/2022	Healthy patient - no smoking in last 40 years/diabetes/med conditions/medications
S1	10/10/2022	>1 pack/day for 30 years
S2	10/25/22	>1 pack/day for 40 years
S3	10/19/2022	1 pack/day for 30 years
S4	10/14/2022	1 pack/day for 6 years

Table 2. PDGF Standard Values.

Standard value	Conc pg/mL	Std Avg con (pg/ml)	OD	Ave OD
2000	1920.377	2001.599	0.976	0.997
	2082.821		1.018	
1000	1033.525	999.8335	0.688	0.668
	966.142		0.654	
500	490.02	500.784	0.408	0.414
	511.548		0.421	
250	257.524	248.707	0.246	0.239
	239.89		0.232	
125	122.679	127.7	0.128	0.133
	132.721		0.138	
62.5	64.421	59.7025	0.067	0.062
	54.984		0.057	
31.3	30.013	32.427	0.026	0.029
	34.841		0.032	

Table 3. PDGF Sample Concentration. **H4 outlier values removed from graphs.*

Sample	Wells	OD	Conc	dilution	Total con (pg/ml)
H1	A3	0.148	143.706	20	2874.12
	A4	0.156	152.091	20	3041.82
	A5	0.131	125.764	20	2515.28
H2	B3	0.16	156.113	20	3122.26
	B4	0.181	179.918	20	3598.36
	B5	0.127	121.144	20	2422.88
***H4**	C3	0.806	1358.873	20	27177.46
	C4	1.07	2301.027	20	46020.54
	C5	0.809	1366.378	20	27327.56
H5	D3	0.116	110.531	20	2210.62
	D4	0.105	99.375	20	1987.5
	D5	0.066	62.915	20	1258.3
S1	E3	0.14	134.711	20	2694.22
	E4	0.127	121.246	20	2424.92
	E5	0.113	107.244	20	2144.88
S2	F3	0.405	486.149	20	9722.98
	F4	0.352	403.451	20	8069.02
	F5	0.224	230.064	20	4601.28
S3	G3	0.399	476.214	20	9524.28
	G4	0.391	463.367	20	9267.34
	G5	0.34	385.014	20	7700.28
S4	H3	0.224	230.43	20	4608.6
	H4	0.183	181.735	20	3634.7
	H5	0.218	223.142	20	4462.84

Table 4. VEGF Standard Values.

Sample	Standard Value pg/mL	BackCalcConc	Wells
01	1500.000	1137.297	A1
		2112.290	A2
02	750.000	799.302	B1
		676.881	B2
03	375.000	385.288	C1
		394.084	C2
04	188.000	187.350	D1
		169.533	D2
05	93.800	99.157	E1
		91.985	E2
06	46.900	46.799	F1
		47.385	F2
07	23.400	25.411	G1
		23.866	G2

Table 5. VEGF Sample Concentration

Sample	Wells	OD	Conc	Dilution	Conc
H1	A3	0.079	13.532	20	270.64
	A4	0.089	19.615	20	392.3
	A5	0.087	18.39	20	367.8
H2	B3	0.141	46.506	20	930.12
	B4	0.155	53.414	20	1068.28
	B5	0.151	51.305	20	1026.1
H4	C3	0.146	49.23	20	984.6
	C4	0.14	46.017	20	920.34
	C5	0.144	48.114	20	962.28
H5	D3	0.089	19.15	20	383
	D4	0.107	29.14	20	582.8
	D5	0.106	28.606	20	572.12
S1	E3	0.08	13.909	20	278.18
	E4	0.079	13.28	20	265.6
	E5	0.082	15.026	20	300.52
S2	F3	0.245	94.589	20	1891.78
	F4	0.219	83.147	20	1662.94
	F5	0.237	90.996	20	1819.92
S4	G3	0.192	70.461	20	1409.22
	G4	0.158	54.75	20	1095
	G5	0.154	53.032	20	1060.64
S4	H3	0.248	96.068	20	1921.36
	H4	0.23	88.206	20	1764.12
	H5	0.183	66.673	20	1333.46

Table 6. TGF Standard Values.

Sample	Standard Value pg/mL	BackCalcConc	Wells
01	2000.000	2184.919	A1
		1831.206	A2
02	1000.000	1219.740	B1
		821.422	B2
03	500.000	558.574	C1
		418.230	C2
04	250.000	257.482	D1
		251.885	D2
05	125.000	161.403	E1
		122.816	E2
06	63.000	110.682	F1
		35.501	F2
07	31.000	Range?	G1
		Range?	G2

Table 7. TGF Sample Concentration. Not in detectable range.

Sample	Wells	OD	R	Conc	AvgConc	SD	CV	Dilution	A
01	A3	0.017	R	Range?	Range?	Ran...	Ran...	30.0	
	A4	0.012	R	Range?					
	A5	0.015	R	Range?					
02	B3	0.021	R	Range?	Range?	Ran...	Ran...	30.0	
	B4	0.030	R	Range?					
	B5	0.023	R	Range?					
03	C3	0.011	R	Range?	12.979	0.000	0.0	30.0	
	C4	0.022	R	Range?					
	C5	0.044		12.979					
04	D3	-0.006	R	Range?	Range?	Ran...	Ran...	30.0	
	D4	0.018	R	Range?					
	D5	0.007	R	Range?					
05	E3	0.004	R	Range?	Range?	Ran...	Ran...	30.0	
	E4	-0.006	R	Range?					
	E5	0.005	R	Range?					
06	F3	0.019	R	Range?	Range?	Ran...	Ran...	30.0	
	F4	0.007	R	Range?					
	F5	0.007	R	Range?					
07	G3	-0.005	R	Range?	Range?	Ran...	Ran...	30.0	
	G4	0.010	R	Range?					
	G5	0.025	R	Range?					
08	H3	-0.006	R	Range?	Range?	Ran...	Ran...	30.0	
	H4	-0.009	R	Range?					
	H5	0.019	R	Range?					

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