1-12-2006

An Examination of the Human Fibrinogen-like Protein2: Sequence Variations and Genetic Expression by Human Endothelial Cells

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AN EXAMINATION OF THE HUMAN FIBRINOGEN-LIKE PROTEIN 2: SEQUENCE VARIATIONS AND GENETIC EXPRESSION BY HUMAN ENDOTHELIAL CELLS

by

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Under the Direction of Roberta Attanasio

ABSTRACT

A role for *Fgl2* (fibroleukin) in thrombophilic-associated human fetal loss has been indicated by previous studies. Clotting of vascular vessels of the placenta and fetus interferes with adequate blood circulation.

The human *Fgl2* gene was sequenced with identification of six SNPs, suggesting an association with a population of women suffering from recurrent fetal losses. A small sample size however, prevented precise statistical analyses of this association. *In vitro*, human endothelial cells (HUVECs) were not found to constitutively express *Fgl2*, but were shown to up-regulate its expression when challenged with IFN-γ and TNF-α. Interestingly, TNF-α was only shown to induce expression of *Fgl2* in HUVECs from male donors and not female donors. A larger case-control study is needed to examine the relationship of *Fgl2* with recurrent fetal loss. The role of estrogen in the Th1-induced expression of *Fgl2* by HUVECs should also be examined.

INDEX WORDS: Fibroleukin, Fgl2, thrombosis, recurrent fetal loss, endothelial cells, HUVEC, Th1 cytokines, IFN-γ, TNF-α

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

In the College of Arts and Sciences

Georgia State University

2005

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Office of Graduate Studies
College of Arts and Sciences
Georgia State University
December 2005

Dedication

This thesis is dedicated to my loving family: Robert, Erin, and William Pyle
and to my incredibly supportive parents, William and Elberta Jenkins.
I could not have accomplished all that I have in life without you.

Acknowledgements

I would like to thank Dr. Craig Hooper and the Centers for Disease Control and Prevention Division of Hereditary Blood Disorders for the use of laboratory facilities and general support for this research activity. I would also like to thank Dr. Christine DeStaercke for her continuing encouragement and faith in my abilities, without which I would not have been "properly" motivated to complete this project before the birth of William.

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LIST OF ABBREVIATIONS

1. Fgl2: fibrinogen-like protein 2
2. mRNA: messenger ribonucleic acid
3. Th1/Th2: T-helper cell ½
4. IFN-γ: Interferon gamma
5. TNF-α: Tumor Necrosis Factor alpha
6. IL-2/4: Interleukin 2/4
7. SNPs: single nucleotide polymorphisms
8. HUVEC: human umbilical vein endothelial cells
9. PCR: polymerase chain reaction
10. HCl: hydrogen chloride (hydrochloric acid)
11. BSA: bovine serum albumin
12. dNTPs: dinucleotide triphosphates

INTRODUCTION

Thrombophila is a condition when a patient has a tendency to form blood clots. This can be a life-threatening event if the clots restrict blood flow. Recent research suggests a possible correlation between inherited thrombophila and recurrent fetal loss. Genetic markers for these clotting factors include factor V Leiden, the prothrombin G20210A polymorphism, activated protein C resistance, and protein S deficiency in first trimester losses. Factor V Leiden has been shown to be significantly associated with early (before 13 weeks gestation) as well as late (after 22 weeks gestation) pregnancy losses. Fibrinogen-like protein 2 (*Fgl2)* may be another genetic marker for thrombosis related to recurrent fetal loss.

Fibrinogen-like protein 2 (*Fgl2*, fibroleukin) was first isolated in 1987 by Koyama, et al.[1] from cytotoxic T lymphocytes. *Fgl2* was described as a novel prothrombinase and procoagulant due to its ability to bypass the traditional coagulation pathway.

The coagulation system consists of an extrinsic and intrinsic pathway. The extrinsic pathway is the initiation of a cascade of sequential activation of coagulation factors. The intrinsic pathway employs the use of cofactors that are involved in the maintenance of this clotting cascade. At the heart of this cascade, tissue factor (a procoagulant) initiates the activation of several factors culminating in the activation of prothrombin to thrombin (by factor Xa in the presence of coenzyme Va). Thrombin then cleaves the zymogen fibrinogen to fibrin, which is subsequently cross-linked and becomes the fibrin clot. Fibroleukin was shown to directly cleave prothrombin to thrombin in a murine model for virus-induced fulminant hepatic failure[2,3,and 4].

Studies conducted in mouse model systems demonstrate the role of *Fgl2* in thrombosis during MHV-3-virus-induced hepatitis[5]. mRNA transcripts of *Fgl2* and Fgl2 protein were detected in liver reticuloendothelial cells, followed by fibrin deposition and widespread hepatic necrosis following MHV-3 infection. Neutralizing antibodies to Fgl2 were able to prevent this scenario, implicating the role of Fgl2 in the fibrin deposition and consequent hepatic pathology. Now linked to thrombosis, Fgl2 was evaluated for a possible role in fetal loss.

Interference of blood supply to the fetus through clotting of the placental/fetal vessels is caused by proteins or cytokines that are produced by immune cells within the lining of the uterus. Initial exposure to pro-inflammatory cytokines is necessary to stimulate invasion of the blastocyst and formation of new blood vessels at the time of implantation. Prolonged exposure of pro-inflammatory cytokines to the pregnancy is detrimental. For pregnancy to be successful, a change in balance of secretion of cytokines from pro-inflammatory to anti-inflammatory cytokines must occur (shift from a Th1 to a Th2 response). During idiopathic recurrent miscarriage, studies point to a Th1 response dominating over a Th2 response. This failure to shift from a Th1 to a Th2 response in early pregnancy (pro-inflammatory to anti-inflammatory cytokines) is known to be detrimental to pregnancy.

Loss of pregnancies in mice after IFN-γ injections has been demonstrated[6]. The same study also showed the ability of IFN-γ to up-regulate the expression of *Fgl2* in the basal uterine deciduas of mice and went on to show that the inhibition of Fgl2 prothrombinase activity was able to prevent cytokine-induced fetal loss normally characterized by fibrin deposition.

Other studies provide additional support to the hypothesis that Th1 cytokines up-regulate the expression of *Fgl2*. One study demonstrates the ability of IL-2 to up-regulate *Fgl2* expression in a murine macrophage cell line, whereas IL-4 (a Th2 cytokine) did not increase Fgl2 production by the mouse macrophages[7]. TNF-α treatment of cultured porcine endothelial cells was also shown to increase the expression of porcine *Fgl2*[8]. These studies provide evidence that *Fgl2* expression is up-regulated by cytokines released during a Th1-type response.

Promoter, intron, and exon regions within the human *Fgl2* gene have been identified, along with different polyadenylation addition signals, which give rise to two mRNA species[7]. Despite the work completed on mouse *Fgl2*, little has been published regarding the regulation of the human gene and its characterization for polymorphisms.

Constitutive expression of human *Fgl2* has been shown in $CD4^+$, $CD8^+$, and cells of the small intestine[9,10]. Conditional expression has been demonstrated by the increased presence of *Fgl2* mRNA seen in endothelial cells and macrophages in patients with acute and viral hepatitis[5]. Uterine tissues taken from fetal losses during the first trimester also show an increased expression of *Fgl2* in trophoblast cells[6].

Estrogen has long been known to have a protective role in thrombophilia environments. The risk of cardiovascular disease in pre-menopausal women is much lower than that of men. The incidence of coronary artery disease and stroke in post-menopausal women (when estrogen levels decrease) rises to equal that of men. Estrogen has also been known to play an important role in the maintenance of a healthy pregnancy. Throughout pregnancy, a steady increase in the amount of estrogen produced by the placenta regulates the production of progesterone over the full term. Estrogen also guides

the maturation of fetal lungs, kidneys, liver, and adrenal glands and plays a role in promoting blood flow within the uterus. The association between estrogen and thrombosis may also indicate a relationship with estrogen and the thrombosis associated with *Fgl2* expression.

In this study, the human *Fgl2* gene was sequenced and variations from the published sequence were identified. The frequencies of these polymorphisms were established in a control population as well as a population of women suffering from recurrent fetal losses. Twelve single-nucleotide polymorphisms (SNPs) were identified in the gene and analyzed for an association with recurrent fetal loss. The expression of *Fgl2* by human umbilical vein endothelial cells (HUVECS) was examined under normal and Th1 cytokine conditions to evaluate if Th1 cytokines influence the expression of the *Fgl2* gene in human endothelial cells. Cells were also challenged with estrogen in combination with Th1 cytokines and evaluated for *Fgl2* expression.

MATERIALS AND METHODS

PCR/Sequencing

A population of human DNA samples was collected consisting of cases and controls. All samples were from female donors and cases were defined as females who had suffered from three or more consecutive miscarriages. PCR primers were designed to amplify the *Fgl2* gene (NCBI accession number AF468959)[11] in 10 short fragments defining nucleotide +1 as the start of translation. Table 1 shows the PCR primers used as well as their nucleotide location in the gene. A PCR master mix was prepared consisting of forward and reverse primers, Amplitaq DNA polymerase (Applied Biosystems), deionized H_2O, and the PCR master mix MasterAmp 2x PCR PreMix J (Epicentre).

Table 2A gives the final concentrations of all ingredients used in the PCR mix. MasterAmp J includes 200µM of each dNTP, buffer, 3.0mM $MgCl_2$, and 8x MasterAmp PCR Enhancer (with Betaine). After adding 8ng/µl DNA template to the reaction mixture, the experiment was run under the thermocycling conditions listed in Table 2B. As shown in Table 2B, the DNA was denatured at 95°C for one minute, annealed at 55°C for one minute, and extended at 72°C for one minute.

An ExoSAP-IT (USB Corporation) clean-up reaction was performed on the PCR product to prepare the samples for a sequencing PCR. Six microliters of ExoSAP-IT was added to 10µl PCR product and was held at 37°C for 30 minutes and then heated to 99°C for 15 minutes as shown in Table 3. The BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) was used to prepare the samples for automated sequencing on the ABI 3100 genetic analyzer. A sequencing master mix was made consisting of Sequencing Mix v.1.1, Sequencing Buffer, 3.2pm/µl sequencing primer, deionized water, and 3µl PCR product. Final concentrations of each additive are listed in Table 4. An additional ethanol precipitation reaction (Table 5) was added to further clean-up the samples before the addition of the 20µl formamide to each sample. Samples were then sequenced on the ABI3100 in a 96-well format.

Table 1 List of PCR and Sequencing Primers

	PCR and Sequencing Primers	Nucleotides
Forward Primer	GTG CTC CAA GTT TGC ATG AC	-1465? -1445
Reverse Primer	CTT CTC TTG CCT ACA GCA TC	-834? -815
Forward Primer	GAT GCT GTA GGC AAG AGA AG	-834? -815
Reverse Primer	CAT CAA ACG CAG TTT GCC AA	-174? -155
Forward Primer	TTG GCA AAC TGC GTT TGA TG	-174? -155
Reverse Primer	TCT TTA GCT CAG AGG ACA GC	+416? +435
Forward Primer	GCT GTC CTC TGA GCT AAA GA	+416? +435
Reverse Primer	GGC ATG CTA AAA CTC TCC CC	1186? 1205
Forward Primer	GGG GAG AGT TTT AGC ATG CC	1186? 1205
Reverse Primer	TCA GAC AGA TAT TTA CTC CC	1876? 1895
Forward Primer	GGG AGT AAA TAT CTG TCT GA	1876? 1895
Reverse Primer	GTA GTG AGG CCT ACC TAT AC	2496? 2515
Forward Primer	GTA TAG GTA GGC CTC ACT AC	2496? 2515
Reverse Primer	GCC ATT ATA GTT ACC AAC GT	3186? 3205
Forward Primer	ACG TTG GTA ACT ATA ATG GC	3186? 3205
Reverse Primer	AAC TCC TGA CCT CAA GTG AT	3896? 3915
Forward Primer	ATC ACT TGA GGT CAG GAG TT	3896? 3915
Reverse Primer	CCC TGG TCT TCA CAG TTA CA	4446? 4465
Forward Primer	TGT AAC TGT GAA GAC CAG GG	4446? 4465
Reverse Primer	ATA AGC AAG CTA CCA GCC AG	4816? 4835

Table 2 PCR Protocol

A. PCR Mix		**B. PCR Conditions**		
Forward Primer (50pm/µl)	.25µl	**Temp.(C) /Time (Min)**		
Reverse Primer (50pm/µl)	.25µl	95 C	1:00	
Master Amp J (2X)	12.5µl	55 C	1:00	x 35 cycles
Taq Polymerase (5U/µl)	.125µl	72 C	1:00	
d-Water	10.875µl	72 C	7:00	
Total Rxn	**24.0µl Mix + 1.0µl (8ng/µl) DNA sample**	4 C	Hold	

Table 3 ExoSAP-IT Reaction

Reaction Mixture		Heating Conditions	
ExoSAP-IT	6.0µl	37 C	30 min
PCR Sample	10.0 µl	99 C	15 min
Total Rxn	**16.0 µl**	4 C	Hold

Table 4 Sequencing PCR

Sequencing Mix (v.1.1)	4.0 µl
Sequencing Buffer	2.0 µl
Primer (3.2pm/ul)	1.28 µl
Water	9.72 µl
Total Rxn	**17.0 µl + 3.0 µl PCR product**

Table 5 Ethanol Precipitation Protocol

Add to Sequencing Plate:	
1.5M NaOAc/250mM EDTA	2.0 µl
3M NaOAc + 1mL EtOH	50.0 µl
Vortex Well	
Centrifuge at 2000 x g	30 Minutes
Remove Supernatant	
Add to Sequencing Plate:	
80% EtOH	100.0 µl
Centrifuge at 2000 x g	5 Minutes
Remove Supernatant	

Cell Culture

Three HUVEC cell lines (from two newborn female Caucasian and one newborn male Caucasian) were obtained from Clonetics-Bio Whittaker and subcultured in an EGM-MV (Cambrex) medium containing 10ng/ml human recombinant epidermal growth factor (hEGF), 1 µg/ml hydrocortisone, 50 µg/ml gentamicin, 50 ng/ml amphotericin-B, 3 mg/ml bovine brain extract (BBE) and 5% FBS (fetal bovine serum). At 80-90% confluence, the cells (P_5) were challenged with 10ng/ml of recombinant human IL-2,

10ng/ml recombinant human TNF-α/TNFSF1A, or 10ng/ml recombinant human IFN-γ (R&D Systems). An additional experiment consisted of the addition of 10ng/ml recombinant human TNF-α/TNFSF1A in combination with 10^{-6} molar β-estrogen. IL-2 was reconstituted in 4mM HCl + 1% BSA while TNF-α and IFN-γ were reconstituted in .01M PBS (pH 7.4) + 1% BSA according to the accompanying protocol (R&D Systems). β-estrogen was diluted in 100% ethanol and growth media. Because it is known that IL-2 does not interact with endothelial cells, the addition of IL-2 to the HUVECs was used as a protein control to demonstrate the protein specificity of any response to IFN-γ and TNF-α. To account for any cellular response due to HCl, BSA, PBS buffer or absolute ethanol on the growth conditions, additional control cells were challenged with 4mM HCl + 1% BSA, .01M PBS + 1% BSA, or PBS plus ethanol.

Cells were harvested at 1 hr, 6hr, 24 hr, and 48hr time points and total RNA was collected and purified using the protocol in Table 6. After RNA purification and quantification, the samples were subjected to a reverse-transcriptase enzyme reaction to prepare cDNA using the Taqman® Reverse Transcription Reagents Kit (ABI) following the manufacturer's protocol. As shown in Table 7, the reaction consisted of a master mix containing reverse Transcriptase Buffer (ABI), $MgCl_2$, dNTPs, Random Hexamers (ABI), Rnase I, and reverse transcriptase enzyme. To this mix, 400ng/ul RNA sample was added.

Once cDNA was prepared, the samples were subjected to Real-Time PCR for the detection of genetic expression of *Fgl2* and the housekeeping gene *GAPDH* using the Assays-on-Demand™ Gene Expression Products (TaqMan® MGB probes, FAM™ dye-

labeled) by Applied Biosystems. The set-up for the reactions is shown in table 8A and 8B and each reaction master mix contained Taqman Master Mix (ABI), 20x primer/probe mix, and deionized water. The reaction was run on the Mx3000P Real-Time PCR System (Stratagene). *Fgl2* and *GAPDH* assays were ran on the same plate to control for amplification differences between samples. Control human RNA (Applied Biosystems) was run alongside the experimental samples as a positive control to ensure the success of the *Fgl2* RT-PCR assay.

Table 6 RNA Purification

Add to Cells:		
1. Homogenization	**1.0 ml TRI reagent**	Store at Room Temp. 5 min.
2. RNA extraction	**0.2 ml Choloroform**	Mix Vigorously Store at Room Temp. 15 min. Centrifuge 12,000 g for 8 min.
3. RNA Precipitation	*transfer aqueous phase to newtube add 0.5 ml isopropanol	mix, store at Room Temp 5 min.
4. RNA Wash	1.0 ml 75% EtOH	mix, then centrifuge 7,500 g for 5 min.
5. Solubilization		air dry pellet 5-10 min Resuspend pellet in 80.0 µl RNase-free Water Incubate at 55-60 C for 10 min

Table 7 Reverse Transcriptase Reaction

RT Buffer (10X)	2.0 µl
$MgCl_2$ (25mM)	4.4 µl
dNTPs (10mM, 2.5mM each)	4.0 µl
Random Hexamers (50µM)	1.0 µl
Rnase I (20U/µl)	0.4 µl
RT Enzyme (50U/µl)	0.5 µl
Total Rxn Mix	**12.3 µl + 7.7 µl (400ng/µl) RNA**

Table 8 RT-PCR Protocol

A. Real-Time PCR Set-Up		**B. Real-Time PCR Thermocycling**		
Taqman Master Mix	10.0 µl	**Hold:**	95 C	10 minutes
20x Primer/Probe Mix	1.0 µl	**Cycling:**	**55-60 cycles**	
Water	5.0 µl		95 C	15 seconds
Total Rxn Mix	**16.0 µl + 4.0 µl cDNA product**		60 C	1 minute

DATA ANALYSIS

The sequence data obtained from the ABI 3100 Genetic Analyzer was entered into the software program SeqMan II version 5.06 by DNAStar. Here, the sequence contigs were assembled (based on overlapping sequences) to obtain one single consensus sequence that was compared against the published sequence for human *Fgl2* (accession number AF468959). Frequencies of the variations between samples among cases and controls were analyzed for significance through computation of a Chi-square. The Chi-square was performed by the software program SigmaStat by Jandel Scientific.

Results from the RT-PCR *Fgl2* gene expression study were analyzed by the Mx3000P Real-Time PCR System. The software performed a comparative quantitation based on the generation of a standard curve for relative amounts of template present. *GAPDH* was used as a normalizer to correct for differences in total cDNA input between samples. The Mx3000P software automatically adjusted the levels of *Fgl2* for differences in the levels of *GAPDH* when generating the standard curve. The amplification efficiency was then calculated based on the slope of the standard curve obtained.

QUALITY CONTROL

Sequencing

Pre-PCR work was conducted within UV workstations in a lab separate from the lab used for post-PCR work (thermocycling, clean-up reactions, and sequencing PCR set-up) to avoid possible routes of contamination. Two separate clean-up reactions were used to remove inhibitors that may interfere with the accurate sequencing of the DNA.

Automated sequencing was performed in the forward and reverse directions and variations in individual samples from the published sequence were repeated to correct for any PCR artifacts or mistakes.

Cell Culture/Gene Expression Assay

Each experiment was set-up alongside three control groups for each time point collected. The first control consisted of growth media only (no addition of cytokines or buffers). The second control included the addition of the buffer (PBS or HCl) or PBS buffer with ethanol that was used to reconstitute the cytokine or estrogen used in the experiment. The third control was a protein control (IL-2) for the demonstration of a cellular response due to the specificity of IFN-γ and/or TNF-α. During the RNA→cDNA reverse transcriptase reaction, as well as the Real Time-PCR reaction, non-template controls as well as manufactured human RNA controls were run alongside the experimental samples to diagnose any contamination and evaluate the overall amplification of the reactions. The Real Time-PCR primers were designed to span exons and eliminate amplification of any genomic DNA contamination. The amplification results of *Fgl2* were normalized to the housekeeping gene *GAPDH* to account for any differences due to varying starting concentrations of cDNA. HUVEC cells from three different donors were independently cultured and tested for *Fgl2* expression. To ensure the success of the *Fgl2* RT-PCR expression assay, positive control human RNA was run alongside the experimental samples. The RT-PCR reaction was also performed a minimum of two times on separate days for each donor for a total of seven independent RT-PCR reactions to ensure reproducibility and consistency in the expression of *Fgl2*.

Statistical Analysis

A Chi-square statistical analysis was performed on all sequencing data obtained. The calculations were made using the program SigmaStat v1.01. A *p*-value was calculated to examine whether or not trends in the data were statistically significant. An accurate Chi-square requires all values to be greater than 1 and no more than 20% of values less than 5. A *p*-value of less than .1 was used as a cut-off to determine statistical significance.

RESULTS

Sequencing

Twelve SNPs were identified at the positions located in the gene map in Figure 1. A T to A change was seen in nucleotide -1285, a T to C change at nucleotide -759, a T to A change at -656, an A to G change at -563, a T to C change at -194, a T to C change at +110, a G to A at +157, a T to C at +2124, a G to T at +2672, a C to T at +3627, a C to T at +3868, and a T to G at +4619. These variations were observed in a direct submission of the *Fgl2* genetic sequence to NCBI (AF468959), however there have been no analyses or identification of the SNPs in relation to the +1 translation start site published. This study defines polymorphisms found in relation to the +1 translation start site, lists their position and frequencies (Table 9) and analyzes the variations for an association with women who have suffered from recurrent fetal losses. As seen in Table 9, the frequency of substitutions at nucleotides -1285 and +110 show a statistically significant difference between cases and controls (p=.01 and p=.03 respectively). A trend in the difference

between cases and controls is seen in nucleotides -656, -194, +157, +2124, +2672, and +3868 but due to small sample size, an accurate Chi square analysis could not be performed. No statistical difference was seen in cases and controls for nucleotides -759, -563, +3627, and +4619. A novel variation from the published sequence not previously reported to date is a nucleotide deletion between positions +4612-4619. The reported sequence contains a string of 8 T's while the current study found only 7 T's occurring in every sample sequenced (170 samples). The raw data and allelic frequencies for the occurrence of the variations seen in this study are displayed in Table 10. As listed in the Table, the data is broken into race categories (Caucasian and African American) for cases and controls. All races (Caucasian, African American, Asian, and Hispanic) were included in the total results column for cases and controls. An accurate Chi-square requires all values to be greater than 1 and no more than 20% of values less than 5. Only one of the SNPs, nt. -1285, met this criteria and was determined to be significantly related to recurrent fetal loss (p=.01). The variation at nucleotide position +110 was also calculated to be significant (p=.03), however the power of the test (.6224) was below the desired power of .800, and therefore should be interpreted cautiously. Due to small sample size or frequency of the variant allele, the analysis of the remaining variations by calculation of a p-value from a Chi- square was not accurately determined.

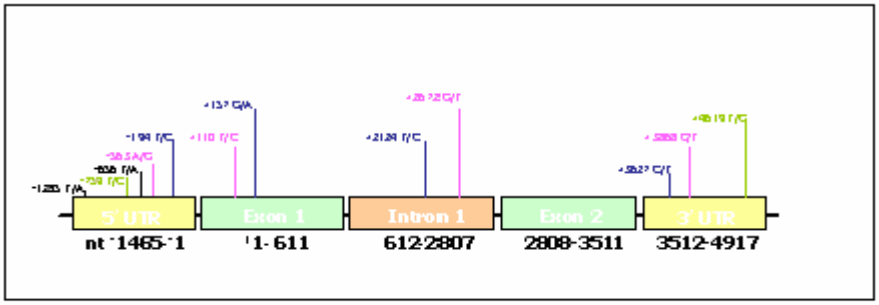

Figure 1 FGL2 SNP Map

Table 9 Frequencies of Variant Alleles

Nucleotide	Controls	Cases	Significance
-1285	**0.52**	**0.65**	**p=.01**
-759	0.01	0.01	
-656	**0.39**	**0.44**	*
-563	0.01	0.01	
-194	**0**	**0.02**	*
+110	**0.06**	**0**	**p=.03**
+157	**0.08**	**0.06**	*
+2124	**0.07**	**0**	*
+2672	**0.02**	**0.04**	*
+3627	0.01	0.01	
+3868	**0.02**	**0.04**	*
+4619	0.01	0.01	

*Chi square analysis not accurate due to small sample size.
(At least 1 value <1 and more than 20% of values less than 5)

**SNPs reported but not found in this study: nt.-210, -165, +1239, and +3124.

Table 10 Raw Sequencing Data and Frequencies

nt -1285 T/A

RAW DATA

	Caucasian		African Americans		Total*	
	Controls (88)	Cases (43)	Controls (36)	Cases (6)	Controls (124)	Cases (49)
TT	12	2	18	1	30	3
TA	38	24	17	4	55	28
AA	38	17	1	1	39	18

Allelic Freq.	Caucasian		African Americans		Total*	.
	Controls (88)	Cases (43)	Controls (36)	Cases (6)	Controls (124)	Cases (49)
TT	0.35	0.33	0.74	0.5	0.46	0.35
AA	0.65	0.67	0.26	0.5	0.54	0.65

*Total Controls and Cases include all races (Caucasians, African Americans, Hispanics, and Asians)

Table 10 continued

nt -759 T/C

RAW DATA

	Caucasian		African Americans		Total*	
	Controls (79)	Cases (50)	Controls (15)	Cases (6)	Controls (96)	Cases (56)
TT	77	49	15	6	94	55
TC	2	1	0	0	2	1
CC	0	0	0	0	0	0

Allelic Freq.	Caucasian		African Americans		Total*	
	Controls (79)	Cases (50)	Controls (15)	Cases (6)	Controls (96)	Cases (56)
TT	0.99	0.99	1	1	0.99	0.99
CC	0.01	0.01	0	0	0.01	0.01

*Total Controls and Cases include all races (Caucasians, African Americans, Hispanics, and Asians)

Table 10 Continued

nt -656 T/A

RAW DATA	Caucasian		African Americans		Total*	
	Controls (79)	Case (39)	Controls (16)	Cases (6)	Controls (97)	Cases (58)
TT	27	11	13	4	40	19
TA	36	18	3	2	39	27
AA	16	10	0	0	18	12

Allelic Freq.	Caucasian		African Americans		Total*	
	Controls (79)	Case (39)	Controls (16)	Cases (6)	Controls (97)	Cases (58)
TT	0.57	0.51	0.91	0.83	0.61	0.56
AA	0.43	0.49	0.09	0.17	0.39	0.44

*Total Controls and Cases include all races (Caucasians, African Americans, Hispanics, and Asians)

Table 10 Continued

nt -563 A/G

RAW DATA	Caucasian		African Americans		Total*	
	Controls (78)	Cases (39)	Controls (19)	Cases (6)	Controls (99)	Cases (58)
AA	78	39	18	5	98	57
AG	0	0	0	1	0	1
GG	0	0	1	0	1	0

Allelic Freq.	Caucasian		African Americans		Total*	
	Controls (79)	Case (39)	Controls (16)	Cases (6)	Controls (97)	Cases (58)
AA	1	1	0.95	0.92	0.99	0.99
GG	0	0	0.05	0.08	0.01	0.01

*Total Controls and Cases include all races (Caucasians, African Americans, Hispanics, and Asians)

Table 10 Continued

nt -194T/C

RAW DATA	Caucasian		African Americans		Total*	
	Controls (73)	Cases (40)	Controls (15)	Cases (5)	Controls (89)	Cases (56)
TT	73	40	15	3	89	54
TC	0	0	0	2	0	5
CC	0	0	0	0	0	0

Allelic Freq.	Caucasian		African Americans		Total*	
	Controls (73)	Cases (40)	Controls (15)	Cases (5)	Controls (89)	Cases (56)
TT	1	1	1	0.8	1	0.98
CC	0	0	0	0.2	0	0.02

*Total Controls and Cases include all races (Caucasians, African Americans, Hispanics, and Asians)

Table 10 Continued

rt +110 T/C

RAW DATA	Caucasian		African Americans		Total*	
	Controls (77)	Cases (45)	Controls (44)	Cases (5)	Controls (133)	Cases (50)
TT	77	45	28	5	117	50
TC	0	0	15	0	15	0
CC	0	0	1	0	1	0

Allelic Freq.	Caucasian		African Americans		Total*	
	Controls (35)	Cases (45)	Controls (38)	Cases (5)	Controls (133)	Cases (50)
TT	1	1	0.81	1	0.94	1
CC	0	0	0.19	0	0.06	0

*Total Controls and Cases include all races (Caucasians, African Americans, Hispanics, and Asians)

Table 10 Continued

nt 157 G/A

RAW DATA	Caucasian		African Americans		Total*	
	Controls (76)	Cases (38)	Controls (45)	Cases (5)	Controls (133)	Cases (43)
GG	63	33	41	5	113	38
GA	11	5	4	0	18	5
AA	2	0	0	0	2	0

Allelic Freq.	Caucasian		African Americans		Total*	
	Controls (76)	Cases (38)	Controls (45)	Cases (5)	Controls (133)	Cases (43)
GG	0.9	0.93	0.96	1	0.92	0.94
AA	0.1	0.07	0.04	0	0.08	0.06

*Total Controls and Cases include all races (Caucasians, African Americans, Hispanics, and Asians)

Table 10 Continued

nt 2124 T/C

RAW DATA	Caucasian		African Americans		Total*	
	Controls (35)	Cases (46)	Controls (36)	Cases (6)	Controls (71)	Cases (66)
TT	35	46	26	6	61	66
TC	0	0	10	0	10	0
CC	0	0	0	0	0	0

Allelic Freq.	Caucasian		African Americans		Total*	
	Controls (35)	Cases (46)	Controls (36)	Cases (6)	Controls (71)	Cases (66)
TT	1	1	0.86	1	0.93	1
CC	0	0	0.13	0	0.07	0

*Total Controls and Cases include all races (Caucasians, African Americans, Hispanics, and Asians)

Table 10 Continued

nt 2672 G/T

RAW DATA	Caucasian		African Americans		Total*	
	Controls (53)	Cases (31)	Controls (7)	Cases (5)	Controls (62)	Cases (49)
GG	51	30	6	3	59	45
GT	2	1	1	2	3	4
TT	0	0	0	0	0	0

Allelic Freq.	Caucasian		African Americans		Total*	
	Controls (53)	Cases (31)	Controls (7)	Cases (5)	Controls (62)	Cases (49)
GG	0.98	0.98	0.93	0.8	0.98	0.96
TT	0.02	0.02	0.07	0.2	0.02	0.04

*Total Controls and Cases include all races (Caucasians, African Americans, Hispanics, and Asians)

Table 10 Continued

nt 3627 C/T

RAW DATA	Caucasian		African Americans		Total*	
	Controls (59)	Cases (36)	Controls (13)	Cases (5)	Controls (72)	Cases (57)
CC	58	36	13	4	71	56
CT	1	0	0	1	1	1
TT	0	0	0	0	0	0

Allelic Freq.	Caucasian		African Americans		Total*	
	Controls (59)	Cases (36)	Controls (13)	Cases (5)	Controls (72)	Cases (57)
CC	0.99	1	1	0.9	0.99	0.99
TT	0.01	0	0	0.1	0.01	0.01

*Total Controls and Cases include all races (Caucasians, African Americans, Hispanics, and Asians)

Table 10 Continued

nt 3868 C/T

RAW DATA	Caucasian		African Americans		Total*	
	Controls (57)	Cases (35)	Controls (7)	Cases (5)	Controls (63)	Cases (55)
CC	56	34	6	5	62	52
CT	1	1	0	0	0	2
TT	0	0	1	0	1	1

Allelic Freq.	Caucasian		African Americans		Total*	
	Controls (57)	Cases (35)	Controls (7)	Cases (5)	Controls (63)	Cases (55)
CC	0.99	0.99	0.86	1	0.98	0.96
TT	0.01	0.01	0.14	0	0.02	0.04

*Total Controls and Cases include all races (Caucasians, African Americans, Hispanics, and Asians)

Table 10 Continued

nt 4619 T/G

RAW DATA	Caucasian		African Americans		Total*	
	Controls (81)	Cases (47)	Controls (20)	Cases (6)	Controls (101)	Cases (69)
TT	81	46	18	5	49	67
TG	0	1	2	1	2	2
GG	0	0	0	0	0	0

Allelic Freq.	Caucasian		African Americans		Total*	
	Controls (81)	Cases (47)	Controls (20)	Cases (6)	Controls (101)	Cases (69)
TT	1	0.99	0.95	0.92	0.99	0.99
GG	0	0.01	0.05	0.08	0.01	0.01

*Total Controls and Cases include all races (Caucasians, African Americans, Hispanics, and Asians)

Gene Expression

Constitutive expression of Fgl2: When grown in EGM-MV growth media, expression of *Fgl2* by HUVECs was not shown at 1, 6, 24, 48, or 72 hours. Figures 2 and 3 show the lack of amplification of these samples, as indicated by the lack of change in fluorescent intensity during the reaction. The human control RNA used as positive controls for the reaction did amplify, indicating expression of *Fgl2* by the samples (plots not shown).

IL-2: As expected, the addition of 10.0ng/ml of IL-2 to the growth media did not induce the expression of *Fgl2* in HUVEC cells from male or female donors. As shown in Figures 4-7, no change in fluorescence was seen, indicating lack of amplification of the samples. Again, the human RNA positive controls amplified, indicating the success of the RT-PCR (plots not shown).

IFN-γ: The analysis of total RNA collected from the cells grown in the presence of 10.0ng/ml IFN-γ showed an increase in the expression of *Fgl2* in a female donor at the 24 hour time point. This expression decreases back to zero at 48 hours. The relative change in fluorescence for these samples can be seen in the linear plot of amplification in Figure 8 and the graph in Figure 9, where an increase in the relative fluorescence indicates amplification (expression) of *Fgl2*. The expression of *Fgl2* grown with IFN-γ in HUVEC cells of a male donor shows an increase of expression at 6 and 24 hours with a decrease of expression at 48 hours , again shown by changes in the relative fluorescent intensity as indicated in Figures 10-11. Positive control RNA was also amplified (plots not shown).

TNF-α: HUVEC cells from a male donor grown in the presence of 10.0ng/ml TNF-α showed an increased expression of *Fgl2* at 24 hours. This expression decreased at 48 hr. Figures 14-15 note the change in relative fluorescent intensity for samples influenced by TNF-α.

The HUVEC cells of a female donor did not show expression of *Fgl2* in any of the time points sampled as shown by the lack of fluorescence seen in Figures12-13.

The gender difference seen in the ability of TNF-α to induce *Fgl2* expression in the HUVEC cells was surprising and led to the investigation of the effect of estrogen on this induction of *Fgl2* expression by TNF-α. As shown in Figure 23, TNF-α was able to induce the expression of *Fgl2* at the 72 hour time-point in HUVECs from a male donor. When estrogen was added to these cells along with TNF-α, the effect was greatly reduced. This is demonstrated by the decrease of fluorescence in the relative quantity chart shown in Figure 23.

Each reaction plate showed an increase in relative fluorescence by the positive control RNA validating the *Fgl2* expression assay (plots not shown). In all cell samples (controls, IL-2, IFN-γ, and TNF-α) presence of RNA/cDNA was shown by the expression of *GAPDH* at all time points collected (Figures 16-22 and 24). Variation in the amplification seen in the GAPDH reactions (some samples were amplified at an earlier cycle than others) can be explained by differences in starting RNA concentrations for the reaction. RNA concentrations were quantified by spectrophotometry, however, limitations in pipetting and the spectrophotometer used may have caused slight variations in concentrations when diluting the RNA for use in the cDNA and subsequent RT-PCR reactions. Using GAPDH as a control gene to assess the expression of RT-PCR assays has recently been under debate due to the variability of its expression levels between the cells of different tissues and while under different experimental conditions[12,13,14]. For this study, however, all RNA samples were prepared from endothelial cells. Additionally, a precise quantification of cDNA expression for *Fgl2* was not needed, as the goal of the project was simply to differentiate between a yes/no result based on the presence or absence of a change in the relative fluorescent intensities obtained for *Fgl2* expression.

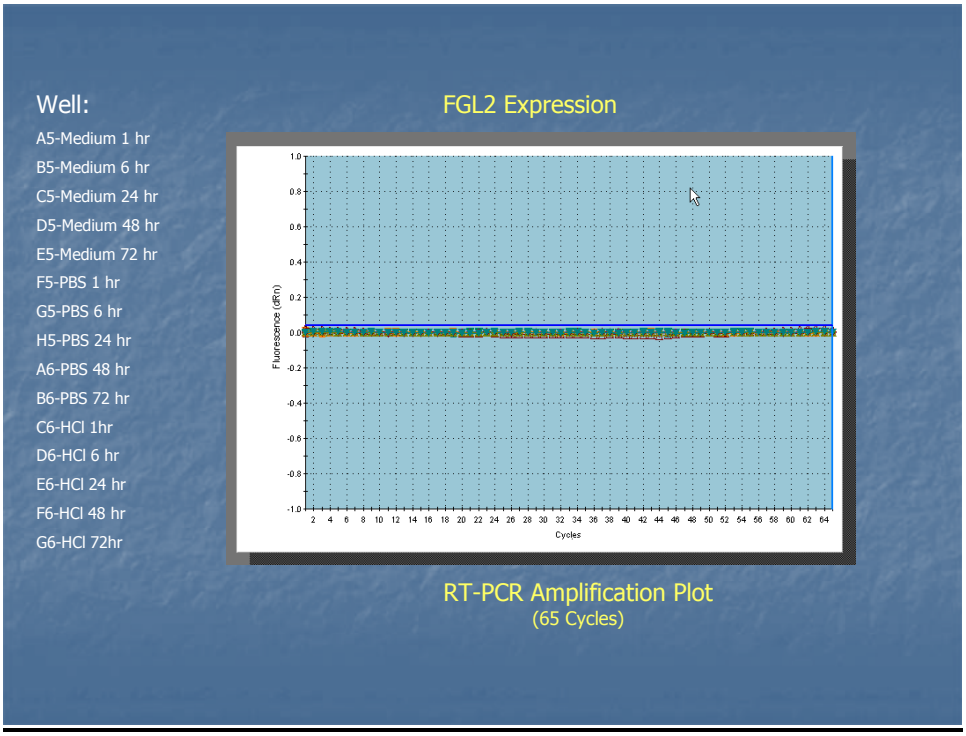

Figure 2 FGL2 Expression in HUVEC Controls: Real-Time PCR Amplification Plot

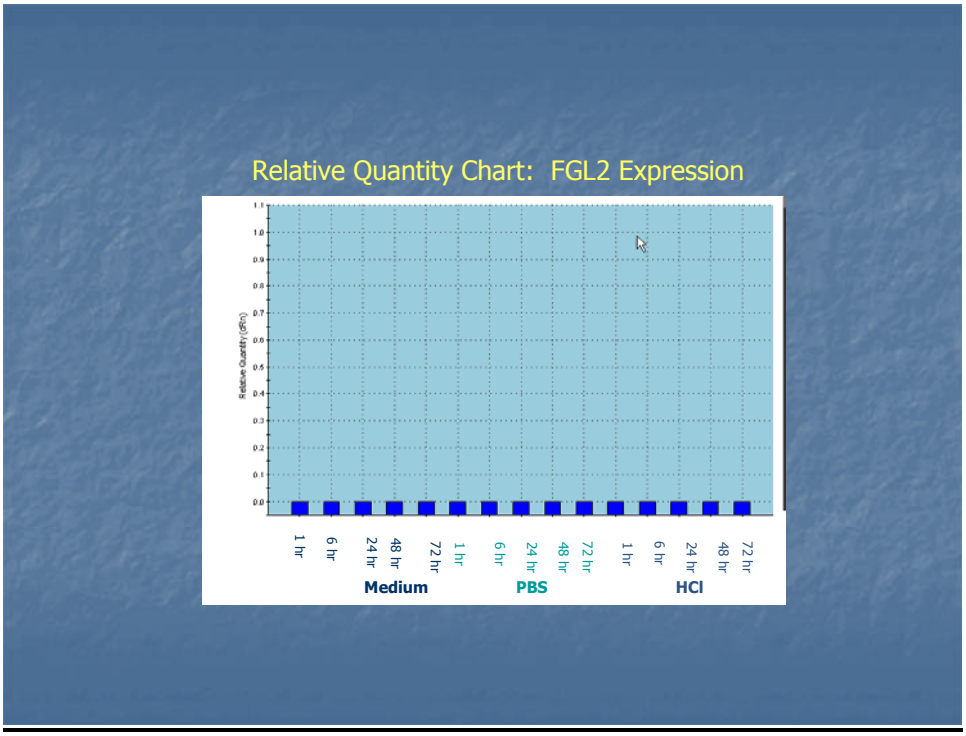

Figure 3 FGL2 Expression in HUVEC Controls: Relative Quantity Chart

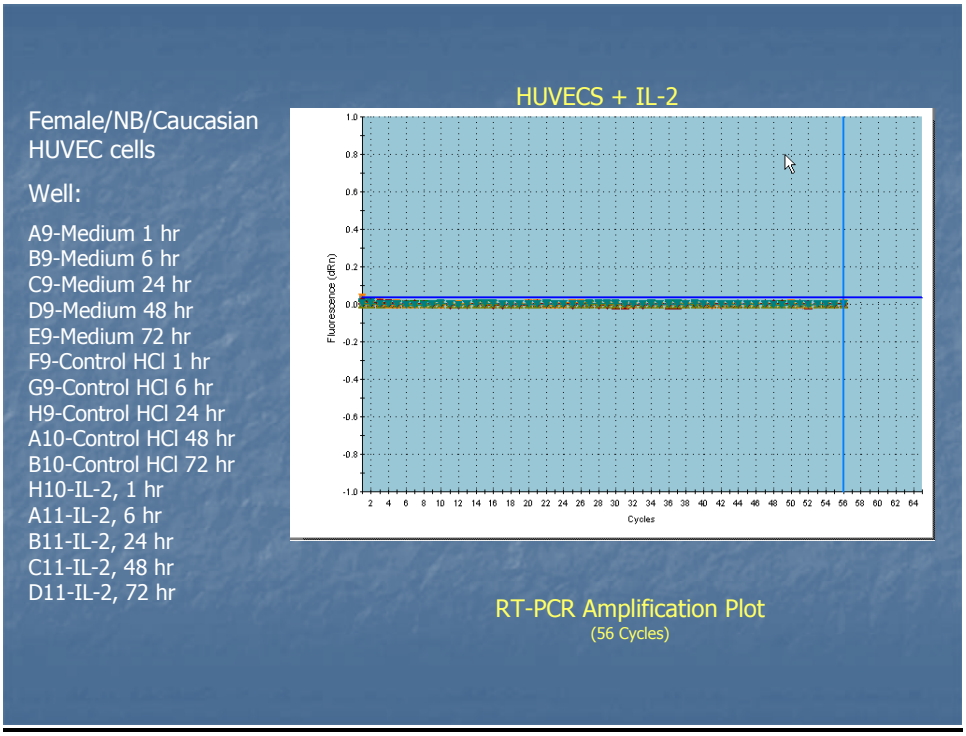

Figure 4 Effects of IL-2 on FGL2 Expression in HUVEC cells, Female donor

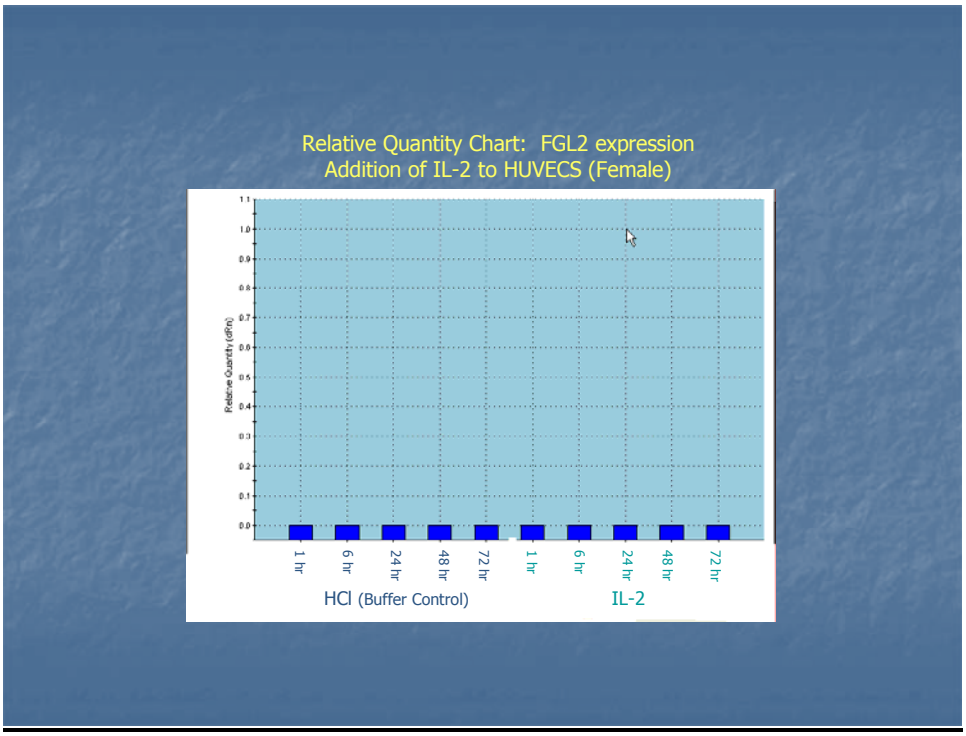

Figure 5 Effects of IL-2 on FGL2 Expression in HUVEC cells, Female Donor

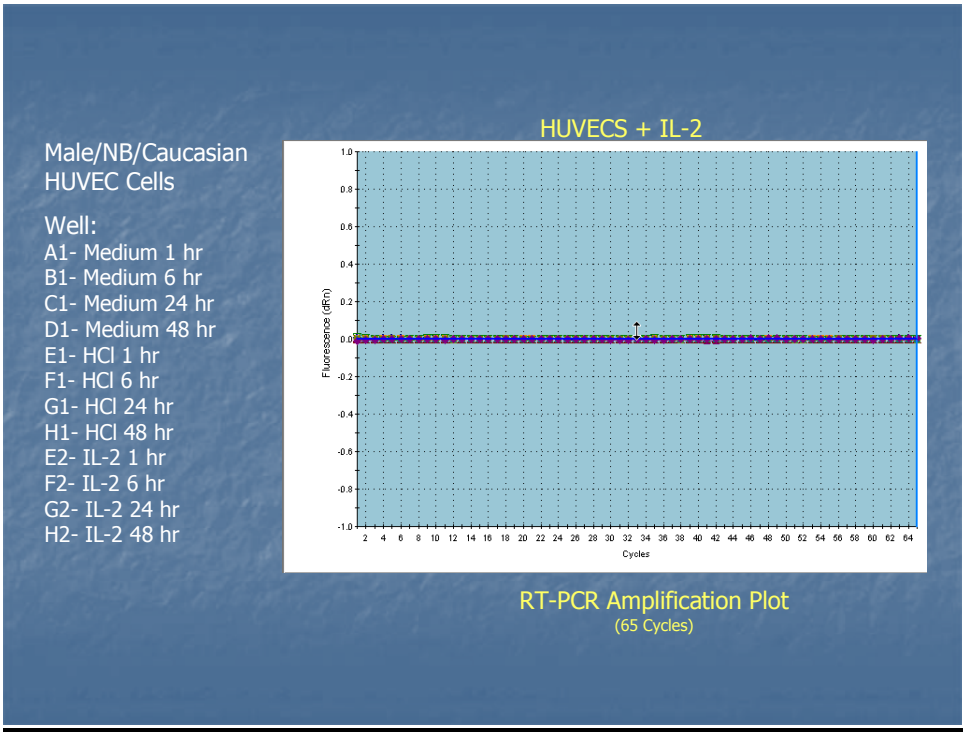

Figure 6 Effects of IL-2 on FGL2 Expression in HUVEC cells, Male Donor

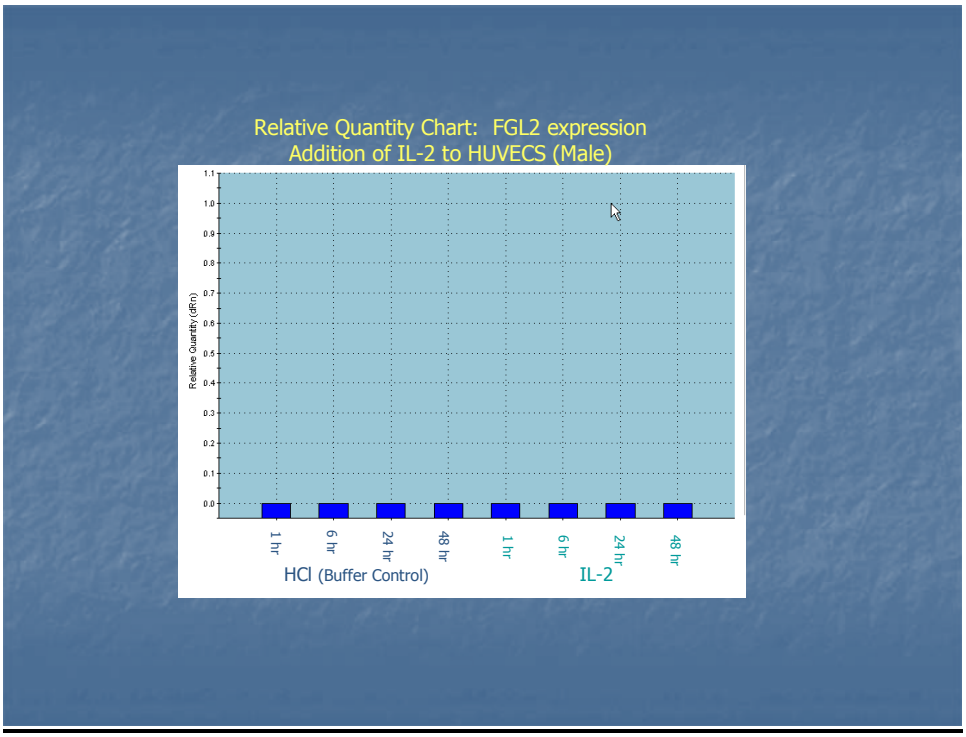

Figure 7 Effects of IL-2 on FGL2 Expression in HUVEC cells, Male Donor

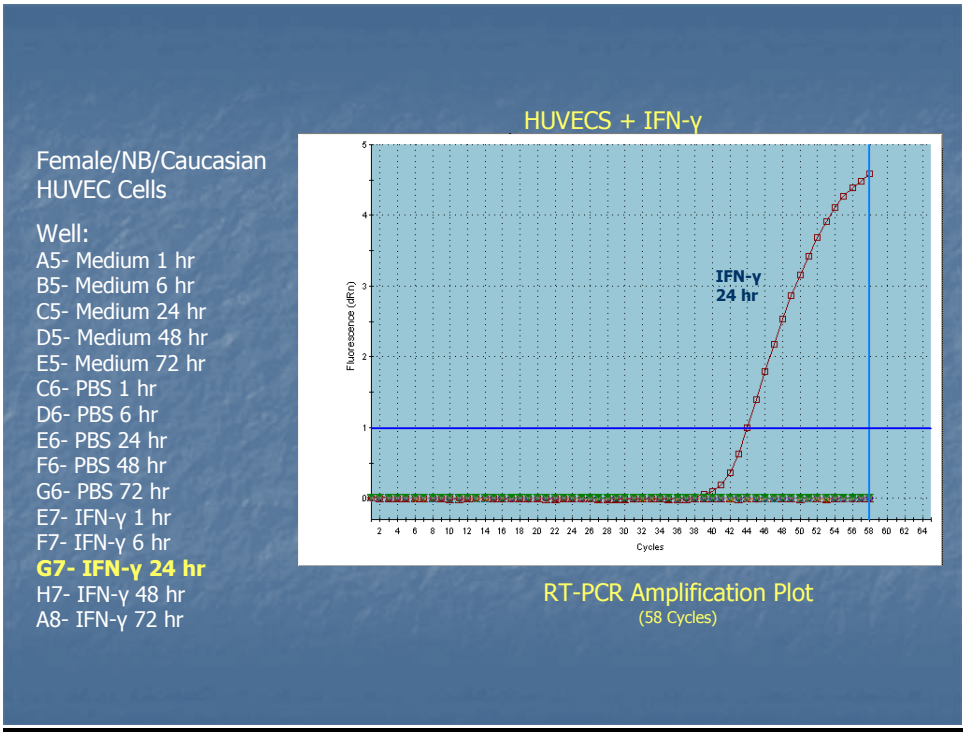

Figure 8 Effect of IFN-γ on FGL2 expression in HUVEC cells, Female Donor

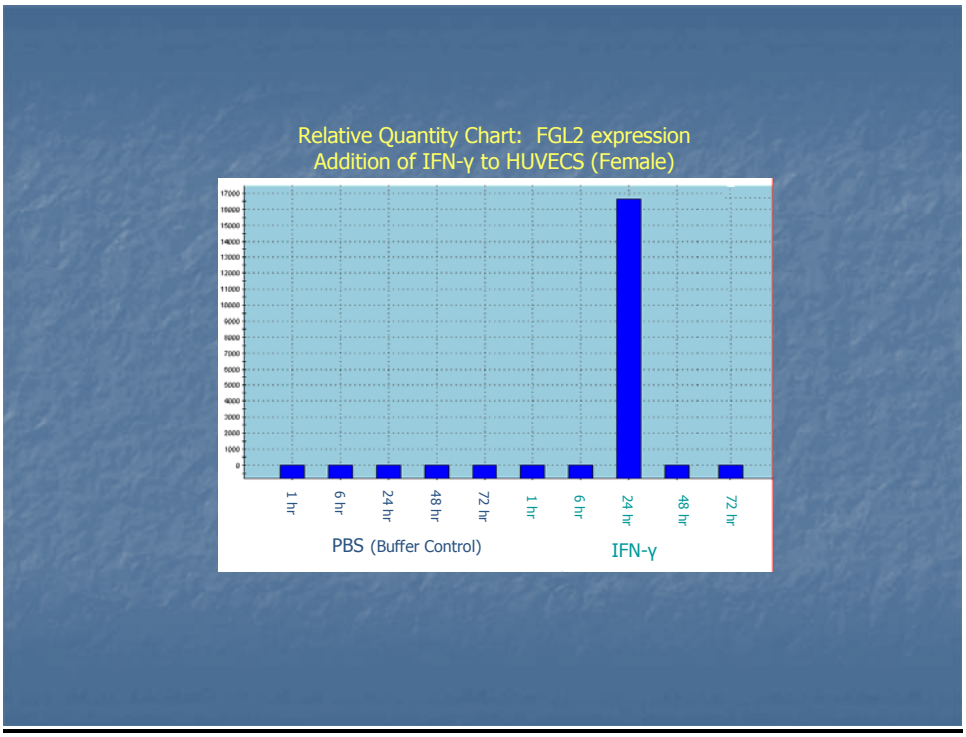

Figure 9 Effects of IFN-γ on FGL2 Expression in HUVEC cells, Female Donor

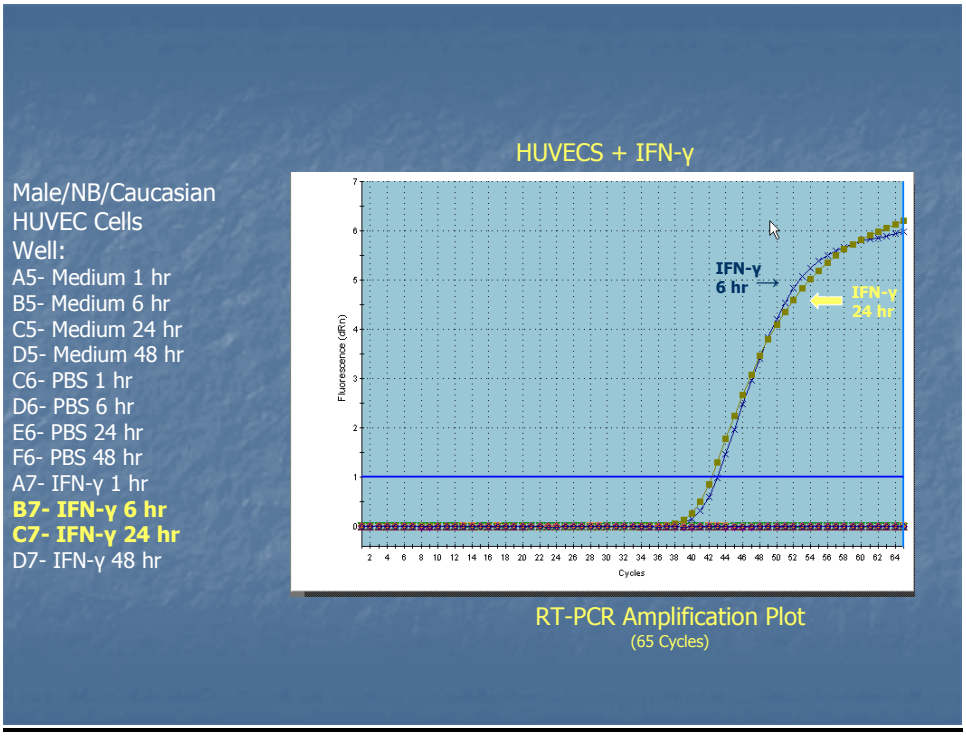

Figure 10 Effects of IFN-γ on FGL2 expression in HUVEC cell, Male Donor

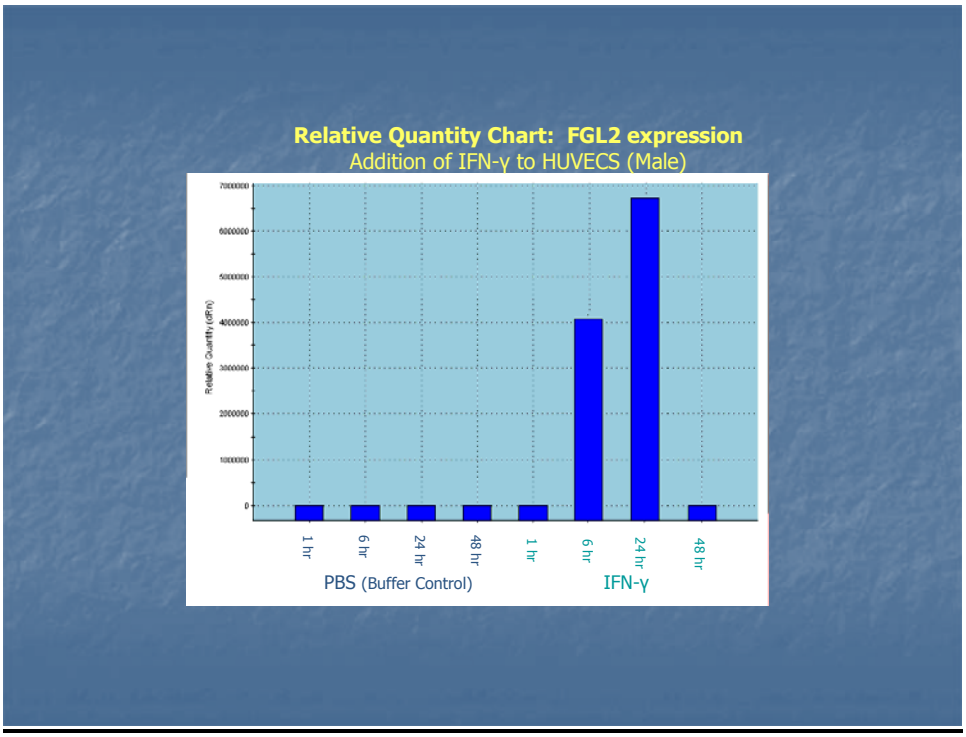

Figure 11 Effects of IFN-γ on FGL2 Expression in HUVEC cells, Male Donor

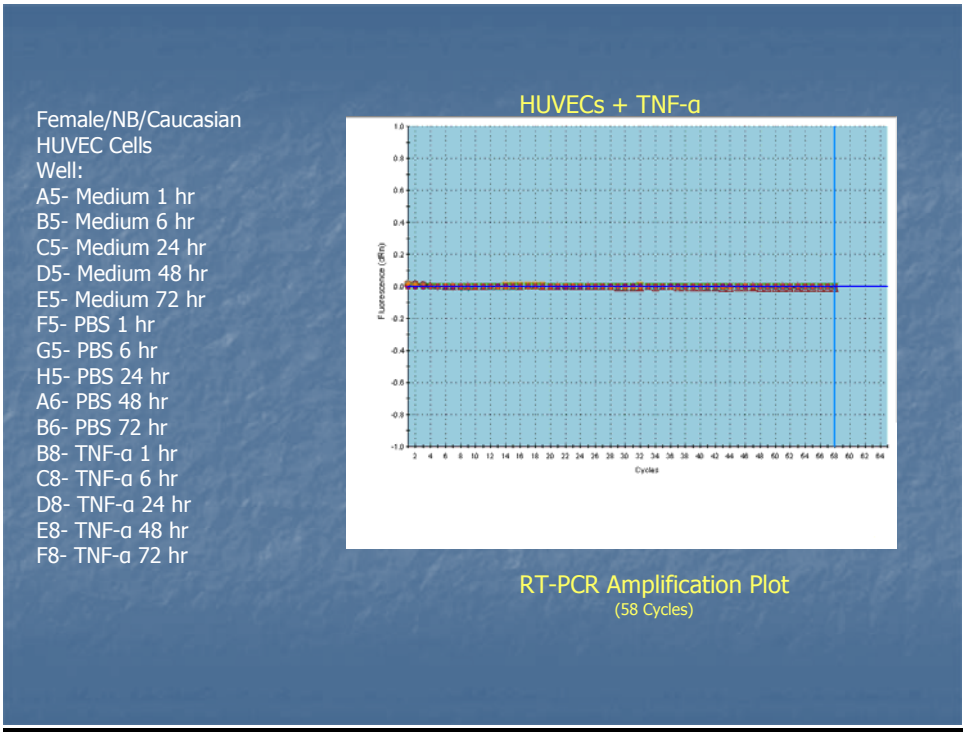

Figure 12 Effects of TNF-α on FGL2 Expression in HUVEC cells, Female Donor

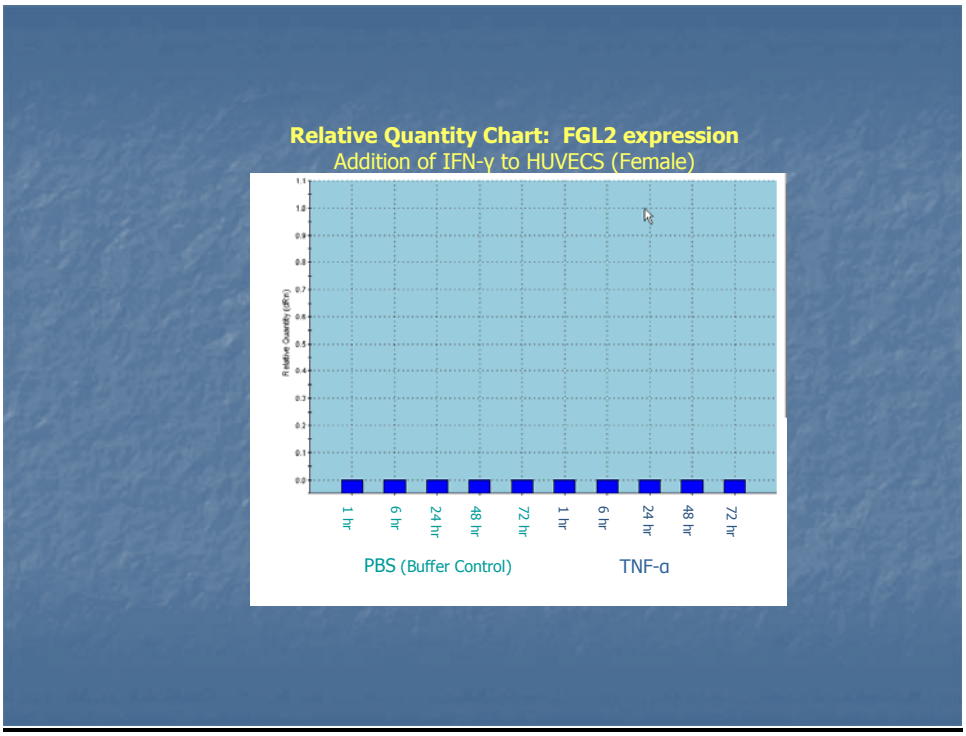

Figure 13 Effects of TNF-α on FGL2 Expression in HUVEC cells, Female Donor

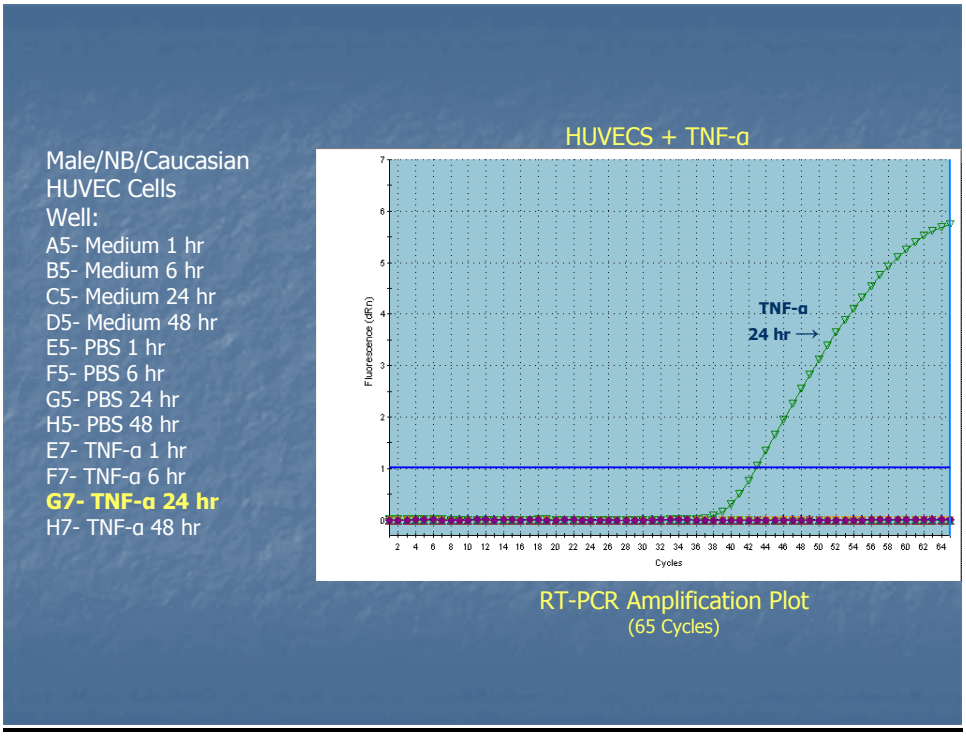

Figure 14 Effects of TNF-α on FGL2 Expression in HUVEC cells, Male Donor

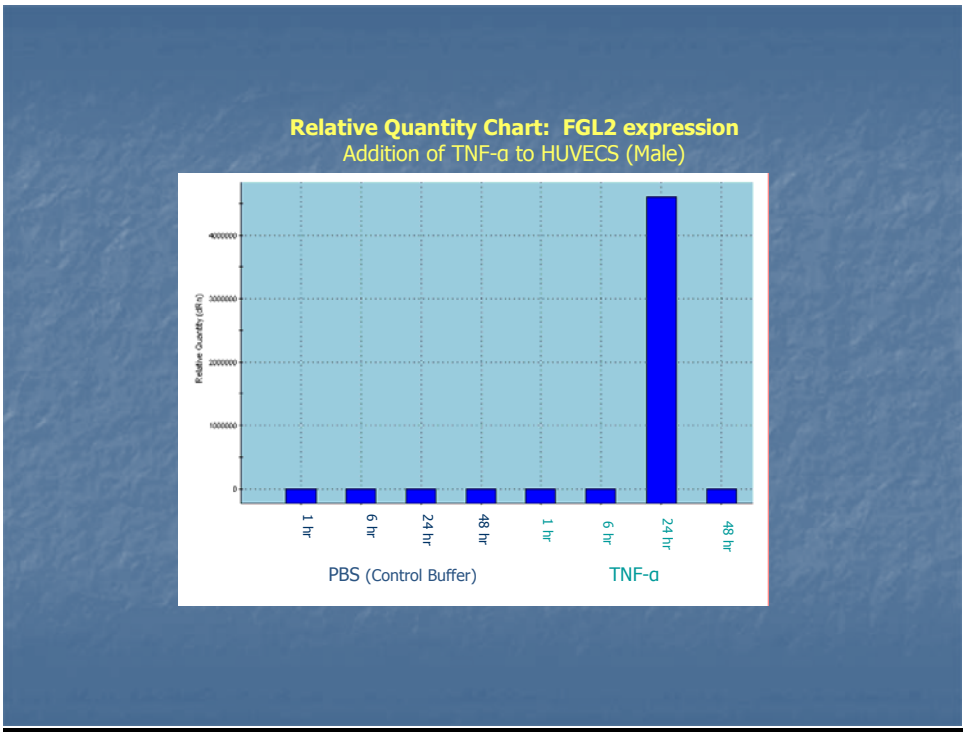

Figure 15 Effects of TNF-α on FGL2 Expression in HUVEC cells, Male Donor

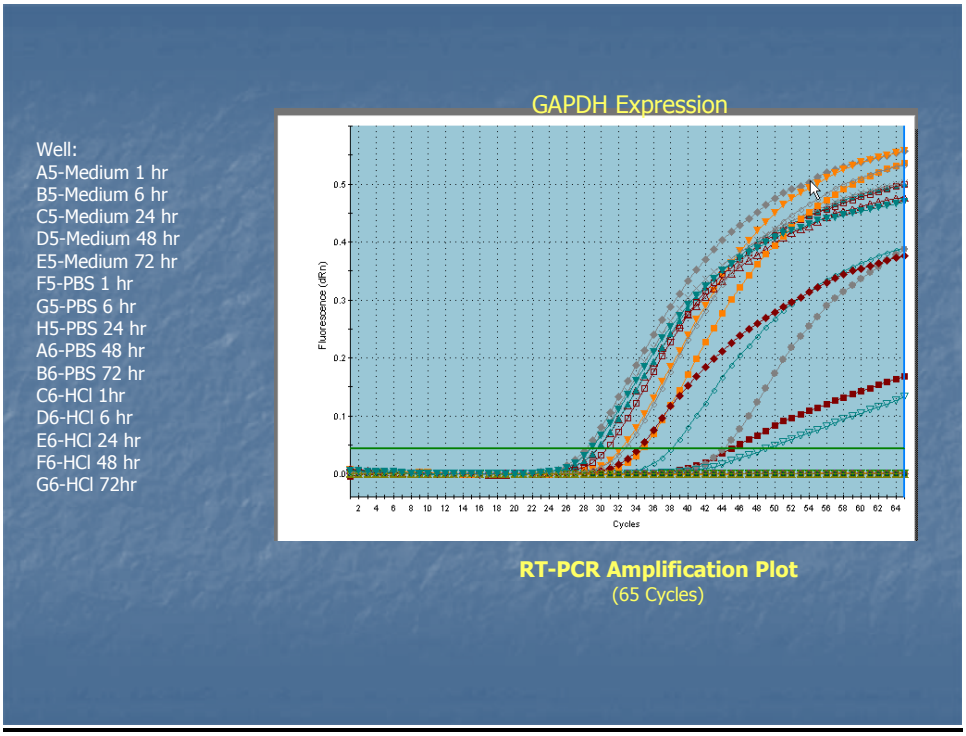

Figure 16 GAPDH Expression in Control HUVEC cell samples

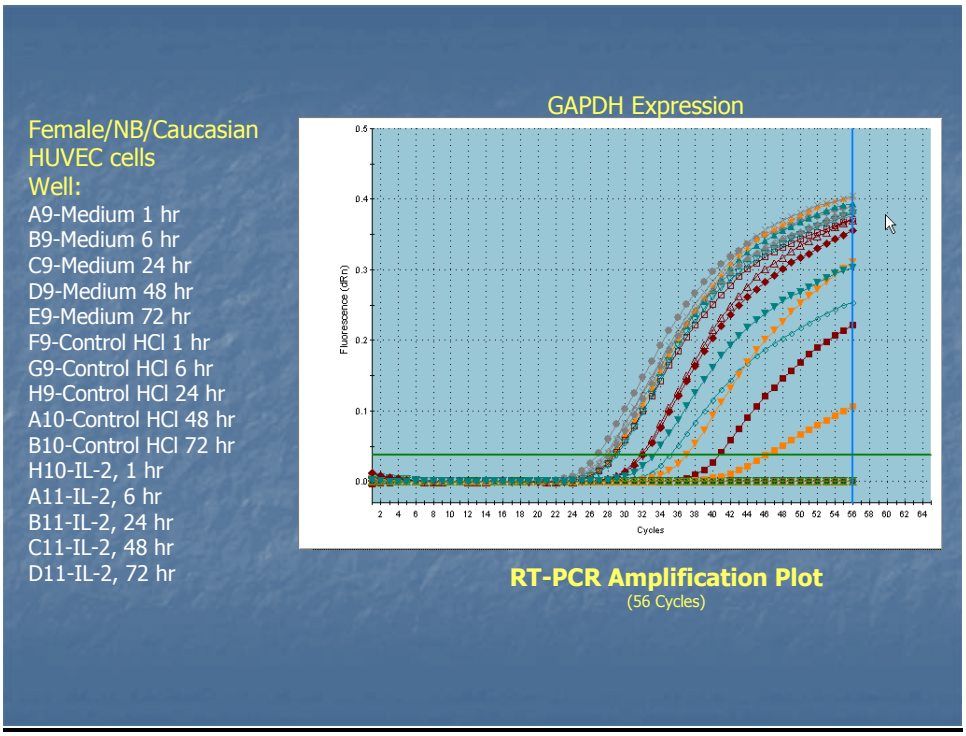

Figure 17 Effects of IL-2 on GAPDH Expression in HUVEC cells, Female Donor

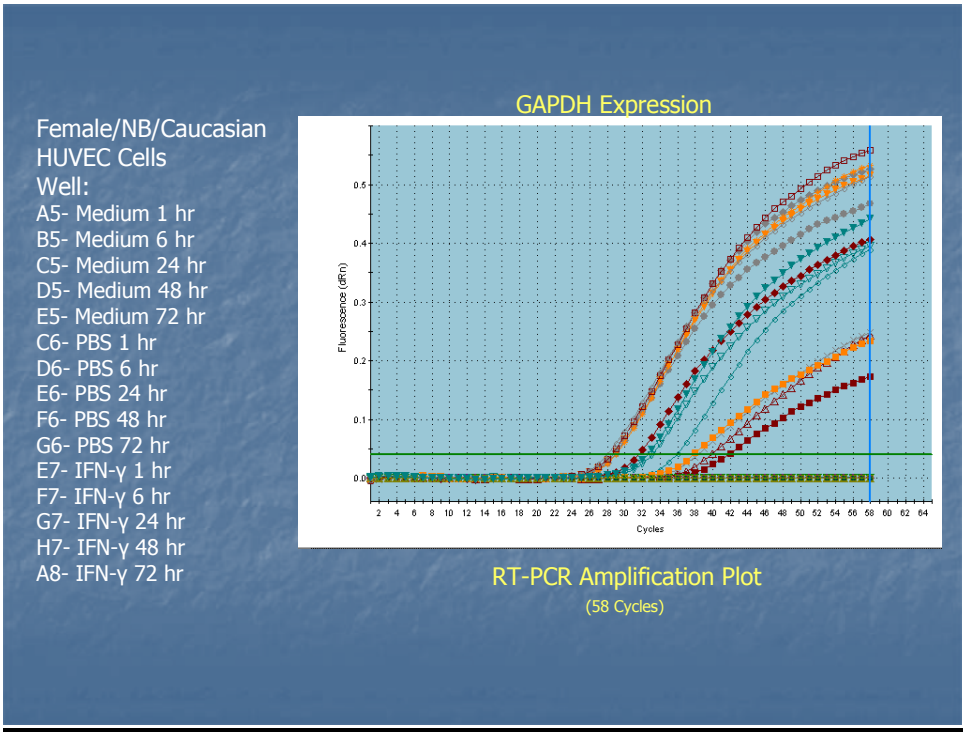

Figure 18 Effects of IL-2 on GAPDH Expression in HUVEC cells, Male Donor

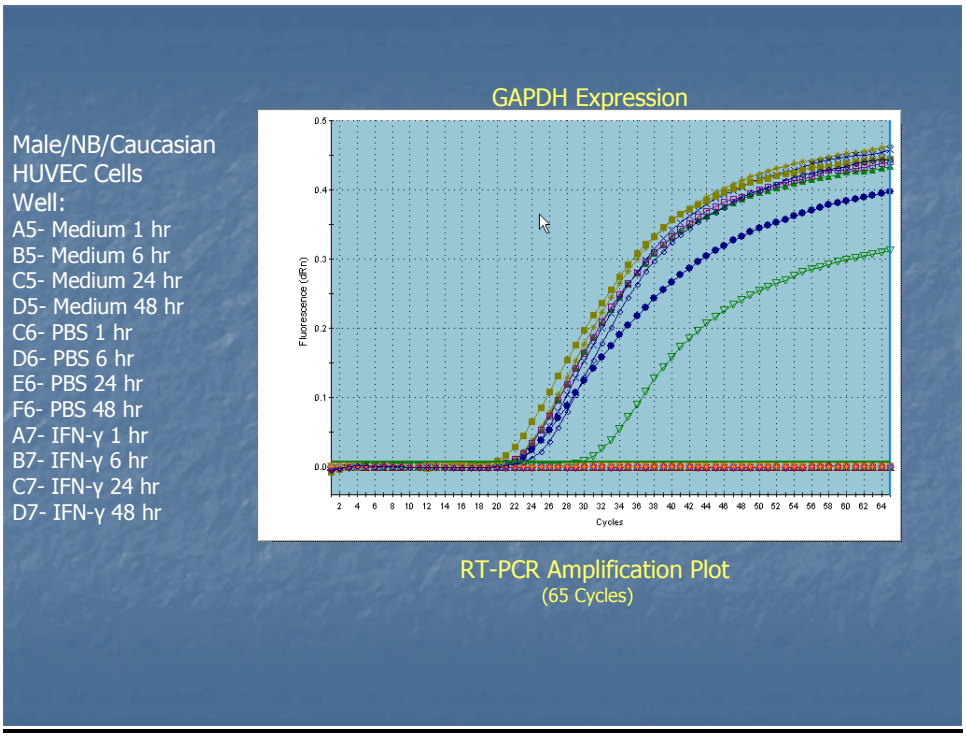

Figure 19 Effects of IFN-γ on GAPDH Expression in HUVEC cells, Female Donor

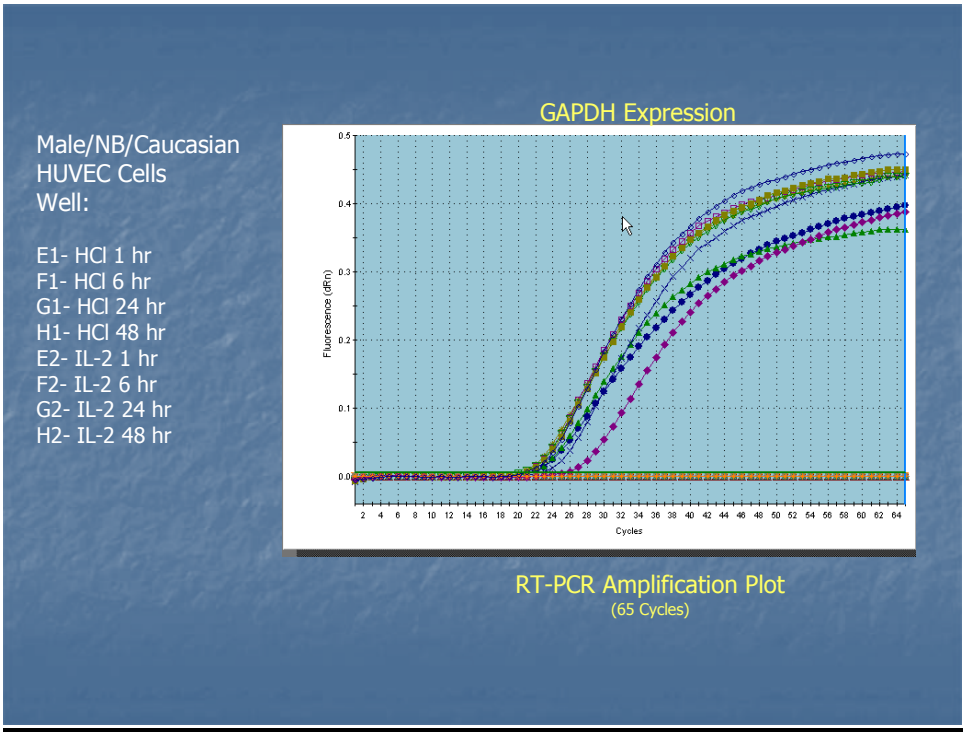

Figure 20 Effects of IFN-γ on GAPDH Expression in HUVEC cells, Male Donor

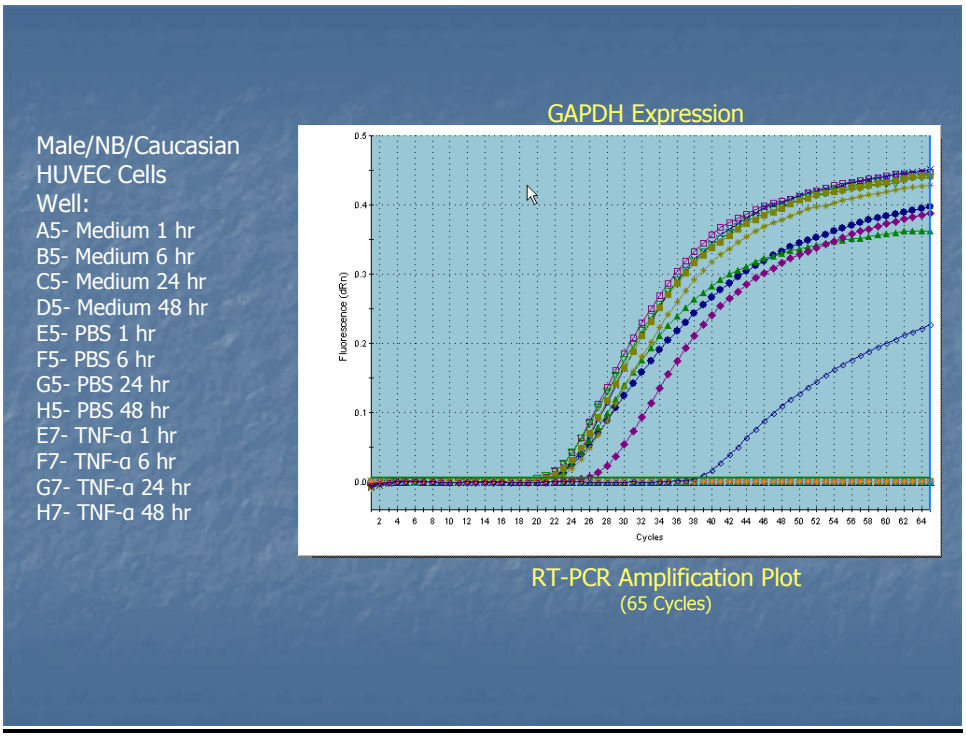

Figure 21 Effects of TNF-α on GAPDH Expression in HUVEC cells, Female Donor

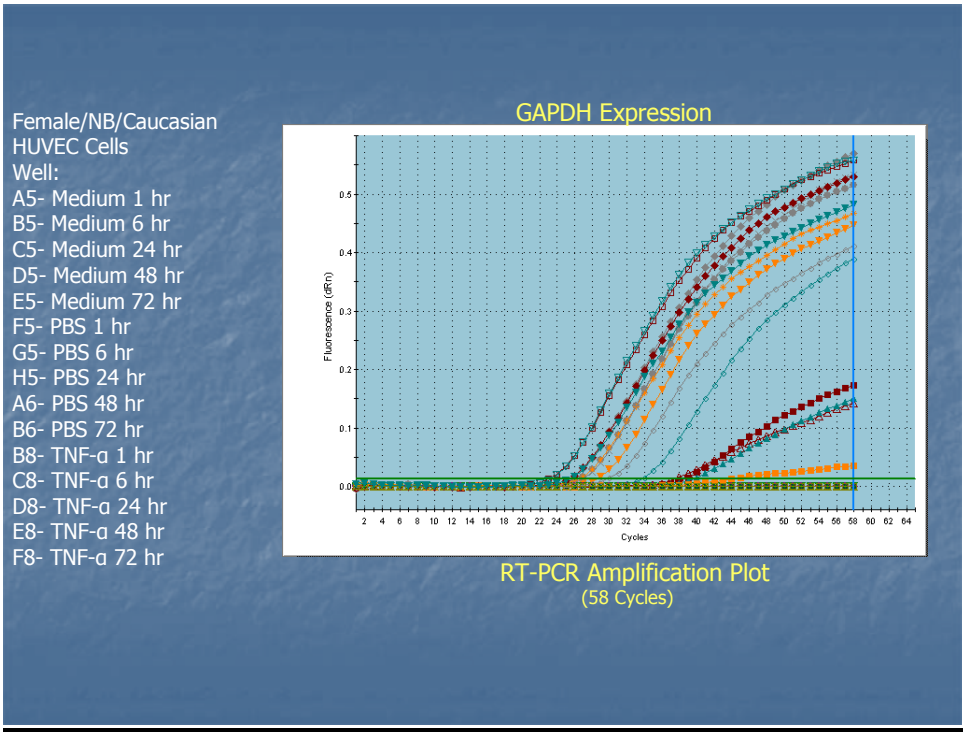

Figure 22 Effects of TNF-α on GAPDH Expression in HUVEC cells, Male Donor

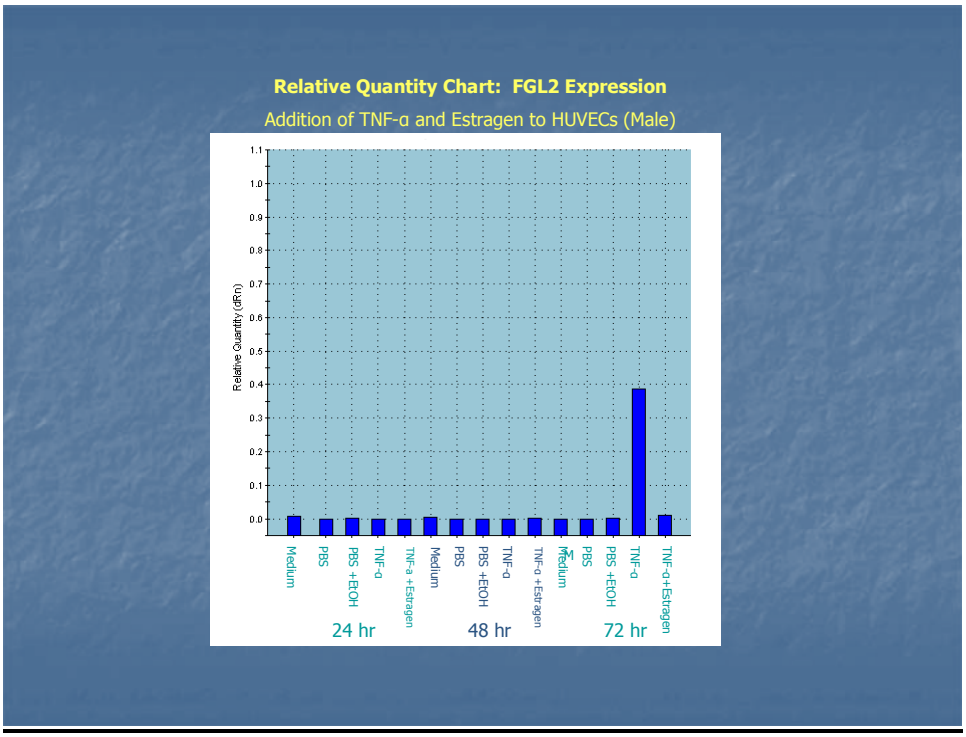

Figure 23 Effects of the addition of Estrogen to TNF-α induced expression of FGL2

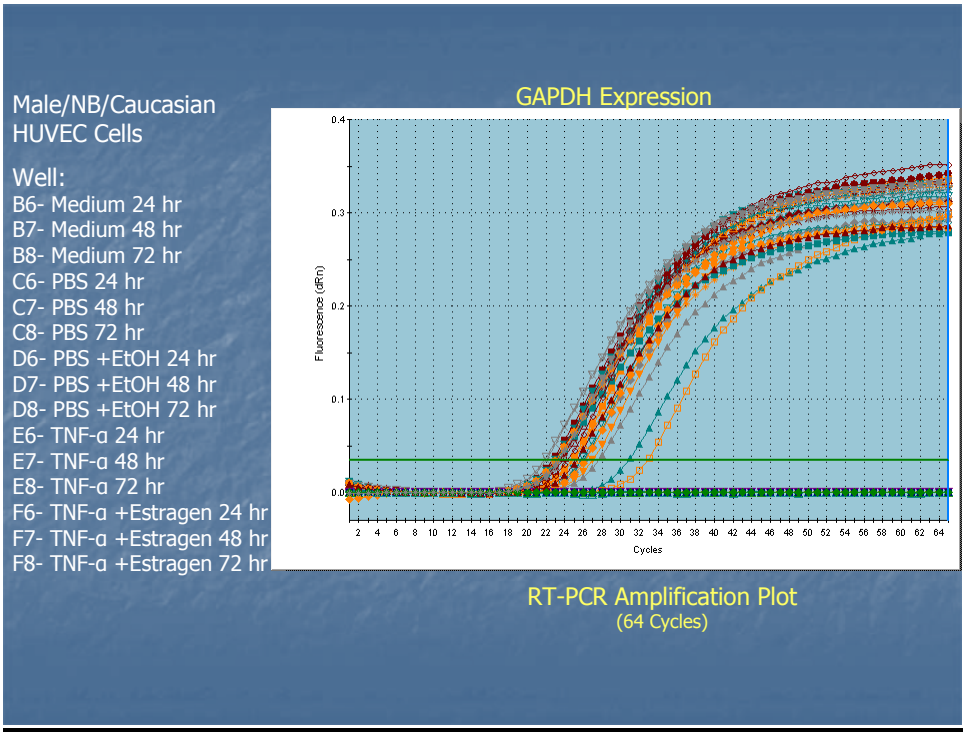

Figure 24 Effects of TNF-α and Estrogen on GAPDH Expression

DISCUSSION

The T→A substitution at nucleotide -1285 is located 30 bp downstream of a conservative MIR (Mammalian Interspersed Repeat) region located in the 5' UTR. MIRs have been implicated in the expression of several mammalian genes, providing alternative splice sites, polyadenylation sites and additional protein-coding information[15,16]. MIR integration has recently been hypothesized to play a role in gene control and evolution[17]. This SNP may or may not affect gene transcription or gene expression. It may, however, be a marker for identifying cases of recurrent fetal loss.

Nucleotide +110 is located in exon 1 of the coding region of FGL2 and involves a T→C substitution. This substitution appears to be a silent mutation, as it changes the reading frame from GAT to GAC, both coding for aspartic acid. This polymorphism was present in the African

American samples tested but was not seen in the White population tested. A low number of African American cases present could account for the apparent significance of the calculated *p*-value between cases and control samples tested. Increasing the study size to include more African American cases may eliminate or reduce the significance of this variation.

The novel variation from the published sequence between nucleotides 4612-4619 (7 T's instead of 8 T's, followed by 8 G's) was seen in every sample tested. Since no representation of the published sequence was discovered in 170 samples for this region of the 3' UTR, a mistake in the original sequence could be possible, since no mention of a nucleotide deletion was made.

Small sample size did not allow the analysis of six additional polymorphisms identified (nt T-656A, T-194C, G+157A, T+2124C, G+2672T, and C+3868T). Differences in the frequency of the variant allele between recurrent fetal loss cases and controls are seen and may suggest a trend for these SNPs. A larger study may indicate a significance that can not be seen in the present study, as many of these SNPs are located within the promoter and other coding regions of the *Fgl2* gene. T-656A is located 20 bp upstream of TCF1 Ets, a transcription factor binding site;[18] the T→C variation at position -194 is located within the promoter region, half-way between binding sites for C/EBP and SP1[18]. T+2124C and G+2672T are located in intron 1 of the non-coding region and flank the ends of another conservative MIR region. Nucleotide +3868 (C→T) is located within the Alu insertion in the 3'UTR of the *Fgl2* gene. Alu insertions have been associated with thrombosis and heart disease when located in the introns of the *TPA* (tissue plasminogen activator) and *ACE* (angiotensin-converting enzyme) genes[19,20].

The G→A variation seen at position +157 has recently been suggested as a marker for severe periodontal disease[21]. Located within the coding region of exon 1, the polymorphism causes a GGG→GAG change in the reading frame which is responsible for an amino acid

change from Gly→Glu (glycine to glutamic acid) which can lead to a significant change in the tertiary structure of a protein. Recent literature supports the hypothesis that chronic oral infection like periodontal disease contributes to pregnancy complications such as preeclampsia, preterm birth, fetal growth restriction, and fetal loss[22,23]. Variations at positions A210G, T165C, T1239C, and C3124T have been reported in the literature for the sequence associated with accession number AF468959, but were not seen in the present study in any sample.

Failure to shift from a Th1 to a Th2-type immune response during early pregnancy is known to be detrimental to a successful pregnancy. This study has shown the ability of the Th1 cytokines IFN-γ and TNF-α to induce the expression of *Fgl2* in HUVECs strengthening the role of *Fgl2* expression in the thrombosis associated with human fetal loss. The lack of an endothelial cell response to the addition of IL-2 further demonstrates the specificity of the response to the Th1 cytokines IFN-γ and TNF-α.

A small gender difference was seen in *Fgl2* expression induced by IFN-γ, in that *Fgl2* was expressed at both 6 and 24 hours in cells from a male donor, and only at 24 hours in cells from a female donor. The sex difference in the expression of *Fgl2* in response to TNF-α was not anticipated and led to the evaluation of the effect of estrogen on this induced expression. As demonstrated, the addition of estrogen and TNF-α to the male donor HUVEC cells was able to greatly reduce the effect seen by TNF-α alone. This supports the large gender difference seen in the expression of *Fgl2* in HUVECs induced by TNF-α.

Clotting of the vascular vessels of the placenta and fetus interferes with adequate blood supply to the fetus. Thrombosis due to the expression of *Fgl2* has been shown to cause fetal loss in mice and increased expression of *Fgl2* in human trophoblast cells in uterine tissue taken from fetal losses suggests a similar role for *Fgl2* in the thrombosis associated with human fetal loss.

The ability of estrogen to eliminate the response of HUVECs to TNF-α in male cells (mimicking the female system) suggests an additional role of estrogen in maintaining a healthy pregnancy. This role could be to protect the pregnancy during times of infection and a Th1-type immune response from activating the prothrombinase *Fgl2* and thus prevent clotting of the placental vessels. This effect might also be involved in the thrombosis associated with cardiovascular disease.

A complex balance between vasodilatory and vasoconstrictory factors and mediators maintains vascular function[24,25]. The incidence of coronary artery disease and stroke in women rises after menopause (when the levels of estrogen decrease), pointing at the important vascular role of estrogen. Coronary artery disease and stroke are the primary causes of death in women after the age of 60. There is about a 50% chance of a post-menopausal woman to develop heart disease in her lifetime, while there is a 30% probability she will die from it[26]. Stroke remains a leading cause of disability and death of women and recent data suggest that one in six women in western countries will die of stroke[27]. The gender difference exhibited by endothelial cells in response to TNF-α and its ability to induce expression of the procoagulant *Fgl2* invites additional studies into this effect in relation to cardiovascular disease. Future research is suggested to compare HUVECs as well as macrophages from pre-menopausal female donors to post-menopausal female donors under conditions favoring the Th1 response (TNF-α) and the induction of *Fgl2* gene expression.

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