Molecular mechanism of the anti-inflammatory effect of fenofibrate

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I. INTRODUCTION

Every thirty seven seconds an American dies from cardiovascular disease (CVD).[1] The estimated cost of health care in 2010 for the one in three Americans affected by cardiovascular disease is over half a trillion dollars.[2] CVD encompasses a wide range of pathophysiologies involving the myocardium and/or its associated blood vessels.[3] In general, CVD involves disease related to atherosclerosis- a disease of the arterial wall characterized by progressive accumulation of lipid and fibrous deposits.[4] CVD is a complex disease influenced by acquired and genetic factors. The high prevalence and the economic impact of this disease drives the need for continuing research in order to alleviate its financial and human cost.

I.1 Atherosclerosis

There are a broad range of risk factors that contribute to the high prevalence of atherosclerosis and subsequent CVD. While some risk factors such as age, sex, family history and genetics cannot be changed, others such as smoking, diet, and physical activity are the first line of CVD prevention.[3] Metabolic abnormalities including obesity, dyslipidemia, and hypertension also put individuals at greater risk. Finally, disease markers such as C- reactive protein (CRP) also play a role in CVD.[5] All of the aforementioned risk factors greatly enhance the probability of developing CVD; the most severe consequences of which are myocardial infarction (heart attack), stroke, and death.[6]

The development and progression of atherosclerosis involves several pathophysiological processes. Atherosclerosis begins with the development of a fatty streak. Low density lipoprotein (LDL) molecules enter the arterial intima and accumulate.[7] The accumulating LDL is oxidized and provokes an inflammatory response

in the vasculature resulting in active recruitment of inflammatory molecules, primarily monocytes and lymphocytes.[8] Upon reaching the intima, monocytes differentiate into macrophages, which then consume oxidized lipid and transform to foam cells. Thus, a fatty streak develops and a thick fibrous cap develops over the legion.[7] Invading inflammatory molecules release inflammatory cytokines and chemokines, which cause the death of smooth muscle cells and the breakdown of collagen. With the progression of this inflammatory state, vessel wall integrity is effectively compromised, resulting in the breakdown of the fibrous cap of the atheroma. The weakened cap is thus more prone to rupture from the pressure of the blood flow across it.[8] A tear in the cap exposes the pro-coagulant subendothelium. A clot can form and block the artery resulting in unstable angina or, if prolonged, a myocardial infarction.[7, 8] Overall, the development and progression of atherosclerosis involves not only lipid accumulation but a strong inflammatory response involving a number of inflammatory cytokines and chemokines [Figure I-1].

I.2 Inflammatory molecules

CVD begins with LDL but it ends when vascular inflammation triggers plaque ruptures. The inflammatory molecules involved in this process are multiple and diverse ranging from cytokines to acute phase proteins. Vascular cell adhesion molecule (VCAM) is a potent cytokine with a strong tie to atherosclerosis. Lipoprotein associated phospholipase A2 (LpPLA2) is an enzyme that acts as an upregulator of inflammation. Transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and acute phase protein C-reactive protein (CRP) also play a role. The

inflammatory markers above all contribute to the strong inflammatory response involved in the progression of atherosclerosis.

I.2.1 VCAM

The VCAM gene encodes a cell surface sialoglycoprotein expressed by cytokine-activated endothelium and mediates leukocyte-endothelial cell adhesion and signal transduction.[9] Investigating the importance of VCAM in animal models of atherosclerosis, Nakashima et al. and Cylbulsky et al. first reported their potential role in the development of atherosclerosis. Using the ApoE -/- knockout model of atherosclerosis, Nakashima et al. found that VCAM-1 staining appeared over the surface of endothelial cells in lesion-prone sites compared to control mice that showed very weak VCAM-1 staining and only where there was altered blood flow.[10] Attempting to elucidate the role of VCAM localization, Cylbulsky *et al*. compared mouse lines with fully functioning VCAM-1 (VCAM +/+) and mice with a mutation in the VCAM-1 gene resulting in drastically decreased production of the protein VCAM-1 (D4D/D4D).[11] The areas of early atherosclerotic regions in VCAM-1 (D4D/D4D) mice were significantly reduced compared to VCAM-1 (+/+) mice. Thus, Nakashima et al. and Cylbulsky et al. showed collectively that VCAM-1 localizes to atherosclerotic lesions and may play a key role in atherogenesis.

I.2.2 LpPLA2

LpPLA2 is an upstream regulator of inflammation and an important inflammation marker in atherosclerosis as it is intimately involved in the formation of the fatty streak.[12] LpPLA2 catalyzes the hydrolysis of the sn-2 fatty acyl bond releasing

lysophatidylcholine (lyso-PC) and oxidized non esterfied fatty acids (NEFA).[13] Both lyso-PC and NEFA upregulate adhesive molecules (eg. VCAM-1, MCP-1) and cytokines resulting in recruitment of monocytes to the intimal space leading to the formation of a fatty streak. Furthermore, lyso-PC and NEFA also release arachidonic acid,[14] a necessary precursor for producing inflammatory leukotrienes as well as prostaglandins.[15] Given the number of inflammatory pathways downstream from the enzyme and its involvement in the formation of the initial fatty streak, it is clear that LpPLA2 is an important inflammatory marker in atherosclerosis.

I.2.3 NFκB

NFκB is a protein complex that acts as a transcription factor which regulates genes that are involved in cell proliferation, cell survival, and the immune response.[16] NFκB has been shown to play a causative role in the inflammatory process and is responsible for the transcription of a multitude of genes that encode pro-inflammatory cytokines and chemokines.[17] NFκB is a heterodimer that is sequestered in the cytoplasm by its inhibitor, IκB. However, when extracellular stimuli activate the IκB kinase complex, the inhibitory IκB protein is phosphorylated, ubquitinated, and subsequently degraded by the 26S proteasome. NFκB is then free to translocate to the nucleus and initiate transcription of pro-inflammatory cytokines and chemokines.[18]

I.2.4 CRP

CRP is an acute phase protein that is upregulated in response to inflammation.[5] The discovery of C-reactive protein (CRP) has allowed inflammation to be measured in a clinical setting. Numerous studies have shown that CRP is associated with CVD and

individuals with elevated levels of CRP are at greater risk for coronary heart disease events.[19-21] It has been shown in the literature that treatments for CVD and atherosclerosis, such as the statins and fenofibrate, reduce CRP levels independent of the lipid lowering mechanisms.[22-24] Overall, the molecular mechanism by which CVD and atherosclerosis drug therapies reduce lipids is well understood, however the molecular mechanism by which these drugs reduce inflammation has yet to be elucidated. There are several drugs on the market for treatment and prevention of cardiovascular disease these include: statins, bile-acid sequesterants, niacin, cholesterol absorption inhibitors, and fibrates.

I.3 Fenofibrate

Fenofibrate, a pro drug that is administered in an inactive form and hydrolyzed to its active metabolite fenofibric acid after absorption by the gut, is used for treatment of hypertriglyceridemia, mixed dyslipidemia and hypercholesteromia.[25, 26] In addition to these lipid modifying effects, fenofibrate also has shown antioxidant effects, anti-inflammatory effects and anti-thrombotic effects, as well as improvements in endothelial function.[27] Thus, fenofibrate provides additional benefits besides its beneficial effect on serum lipid profile.

Mechanistically, fibrates stimulate cholesterol efflux by upregulating proteins involved in reverse cholesterol transport (RCT) and decreasing the production of proteins that interfere with RCT. ATP-binding cassette transporter A1 (ABCA1) is involved in the first steps of RCT by mediating the efflux of phospholipids to lipid poor apolipoproteins (eg. Apoliprotein AI and apolipoprotein AII).[25] Apo AI and Apo AII are

both major components of high density lipoprotein (HDL)[28], (and then form nascent high density lipoprotein). Fibrates induce the expression of the genes of Apo AI and Apo AII thus increasing the serum concentration of HDL level.[29,28,30] Fibrates also decrease production of apolipoprotien C-III, a protein which inhibits triglyceride hydrolysis by lipoprotein lipase (LPL), and upregulates LPL.[29,28] This leads to increased very low density lipoprotein (VLDL) clearance and produces more buoyant LDL particles. Finally, fibrates increase the synthesis of Acyl-CoA synthase which catalyzes the addition of free fatty acid onto Acetyl-CoA.[28] This reduces the levels of free fatty acids in hepatocytes, leading to decreased production of triglycerides and decreased production of VLDL.[30-32] The stimulated expression of Apo A-I, Apo A-II, and ABCA1 all lead to increased production of HDL particles and RCT. While the increase of LPL and Acyl-CoA synthase clear VLDL from the blood stream and decrease its production. The lipid modifying effects of fenofibrate is largely due to the activation of PPAR-α [Figure I-2].

I.4 Peroxisome proliferator-activated receptors

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors of the nuclear hormone receptor superfamily. PPARs form a heterodimer with another nuclear receptor, retinoid X receptor (RXR). The heterodimer of PPAR/RXR binds to a specific DNA sequence element called proliferator response element (PPRE), which lies within the promoter of the genes regulated by PPARs.[33] There are three different isoforms, PPAR-α, PPAR-γ, PPAR-δ; in general PPARs regulate energy homeostasis, as well as the balance between cell proliferation, survival and differentiation. PPAR-α plays a key role in fatty acid transport and oxidation and PPAR-γ is a major regulator of lipid storage in adipose tissue, regulates differentiation of

adipocytes and has anti-inflammatory activities[34] whereas PPAR-δ is involved in brain lipid metabolism, embryo implementation, and anti-inflammatory activities.[32] All isoforms interact with saturated and unsaturated fatty acid ligands. Fenofibrate is a synthetic ligand for PPAR-α and the drug class of thiazolidinedione, also called glitazones, is a ligand for PPAR-γ. There is currently no drug that specifically targets PPAR-δ.[35] PPAR's are nuclear hormone receptors that regulate energy homeostasis and act as agonists for natural and synthetic ligands.

PPAR- δ has shown potential as a possible therapeutic target for CVD and the ability to regulate the other isoforms of PPAR. While there is no drug that specifically targets PPAR –δ, a study by Oliver et. al. treated obese rhesus monkeys with a synthetic PPAR-δ agonist, GW501516, in a dose dependent matter and showed changes in metabolic variables. There was a 79% increase in serum HDL levels, a 56% decrease in triglycerides, a 29% decrease in LDL, and a 48% increase in fasting insulin levels. These results suggest that PPAR-δ agonists may provide a promising therapeutic target for CVD.[36] Furthermore, PPAR-δ has also shown a capacity for regulating PPAR- α and PPAR-γ. Two hybrid assays demonstrated that PPAR-δ represses PPAR-α and PPAR-γ mediated transcription. Competition for DNA binding to PPRE and the recruitment of co-repressors are the underlying mechanism for PPAR-δ-mediated isoform repression.[37] The differential expression of PPAR-δ acts as a gateway receptor to modulate PPAR-α and PPAR-γ. PPAR-δ is emerging as a prospective player in the prevention of CVD.

I.5 Preliminary Data

A recent study in our laboratory reported that fenofibrate treatment induced B-cell CLL/lymphoma 6 (zinc finger protein 51), Bcl-6, and Split ends (Spen) in total RNA in peripheral leukocytes.[38] Nineteen individuals with hypertriglyceridemia (baseline plasma TG levels > 150 mg/dl) were selected from the GOLDN study (Genetics of lipid of lowering drug and diet network). GOLDN subjects went through a 3-week unblinded fenofibrate treatment (160 mg/d). Microarray analysis was performed on Genenews TM platform and paired t-tests were used for comparing before and after treatment gene expression profiles. Bcl-6 showed a 1.8 fold increase and Spen showed a 1.7 fold increase after 3 weeks of fenofibrate treatment. Among the 271 genes that showed different total RNA quantity before and after treatment, Spen and Bcl-6 represented the greatest increase in total RNA.

I.6 B-cell CLL/lymphoma 6 (zinc finger protein 51)

The protein encoded by Bcl-6 is a zinc finger transcription factor that is involved in transcription repression, cytoskeleton regulation, and protein ubquitination/degredation.[39, 40] Bcl-6 is found in low abundance in multiple tissues but highly expressed in mature B-cells.[41] Bcl-6 needs a DNA binding site and a POZ domain for cooperative binding to repress the transcription of the target gene.[42] POZ (Pox virus zinc finger) forms obligate homodimers with other POZ domains[40] and recruits histone deacetylase (HDACs) and other nuclear co-repressers.[43] Bcl-6 is a sequence specific repressor of transcription that interacts with POZ domains of other genes.

Recently, Bcl-6 was found to interact with PPAR-δ in a ligand sensitive manner.[31] Immunoprecipitation of transfected 293 cells (human kidney epithelial cells) expressing

epitope-tagged Bcl-6 and PPAR-δ indicated that the proteins interacted in a complex that was disrupted by PPAR-δ ligand. A mutant PPAR-δ receptor that is deficient in ligand-induced compressor release maintained interaction with Bcl-6 regardless of the ligand status. These results suggest that Bcl-6 and PPAR-δ associate when PPAR-δ is in its repressive mode. Further experiments done by Fan et al. have confirmed the results found by Lee et al. [31] Fan et al. performed a co-immunoprecipitation and confirmed that Bcl-6 interacts with PPAR-δ in human umbilical vein endothelial cells (HUVECs). The addition of ligand reduced the amount of Bcl-6 associated with PPAR-δ, but not the amount of intracellular protein.[44] Both investigations into Bcl-6 provide evidence of its direct interaction with PPAR-δ.

I.7 Bcl-6 Target genes

Bcl-6 binds to specific DNA target sequences within the promoter region of its target gene.[42] NFκB, a protein complex which acts as a transcription factor, is a direct transcription target of Bcl-6 in mature B cell lines, skeletal muscle, thymus, and macrophage lines. In macrophage cell lines, Bcl-6 -/- mice showed an increase in p105/ p50 expression and enhanced NFκB binding activity of p50 homodimer and p50/p65 heterodimer.[45] The data suggest a repressive role for Bcl-6 on NFκB expression.

Apart from NFκB, Bcl-6 may also repress additional inflammatory molecules such as monocyte chemotactic protein 1 (MCP-1) and VCAM . MCP-1 is a chemokine that recruits inflammatory molecules to sites of infection or injury. Toney et al. showed that Bcl-6 directly repressed the transcription of MCP-1 in macrophages.[46] The inhibitory

effect of PPAR-δ agonist on MCP-1 gene expression was proposed to result from the disassociation of Bcl-6 from PPAR-δ after ligand binding[47] [Figure I-3].

While Toney et al. focused on MCP-1, Fan et al. explored the potential for Bcl-6 to repress VCAM-1. Using chromatin immunoprecipitation (CHiP) assays, Fan et al. demonstrated that the PPAR-δ synthetic ligand, GW0742, increased the association of Bcl-6 and the VCAM-1 promoter. This suggests that GW0742 suppresses endothelial activation by initiating the relocation of Bcl-6 from PPAR-δ to the promoter region of pro-inflammatory target genes (eg. VCAM).[44] Toney et al. and Fan et al. demonstrated that Bcl-6 targets inflammatory molecules VCAM-1 and MCP-1, and furthermore, the interaction between Bcl-6 and the inflammatory molecules is mediated by PPAR-δ.[44, 46]

Further supporting its role as an anti-inflammatory protein, Bcl-6 was also found to repress IL-1β – induced sPLA2-IIA gene transcription in vascular smooth muscle cells (VSMC). VSMCs were transciently transfected with the luciferase reporter plasmid containing the sPLA2 promoter. The repressor activity of Bcl-6 was measured by co-transfecting the reporter gene and Bcl-expression vectors into VSMCs. The transfected cells were pre-treated with PPAR-δ agonist for 6 hr and then stimulated with IL-1β for 24 hr. Inhibition of sPLA2 by Bcl-6 vector was strengthened after treatment by PPAR-δ agonist. The data suggest that Bcl-6 functions as a potent repressor for the sPLA2-IIA gene in VSMCs. ChIP experiments were done to confirm that Bcl-6 binds to the endogenous sPLA2 promoter and that PPAR-δ ligands induce that binding.[48] The authors subsequently concluded that Bcl-6 directly targets sPLA2 and is directly influenced by PPAR-δ ligands. Our preliminary study confirmed this finding in 100 individuals of the

GOLDN study: fenofibrate reduced LpPla2 levels especially in individuals with high baseline LpPLA2.

I.8 Spen

Spen is a hormone inducible transcriptional repressor first identified in Drosophila. The human homolog of this gene is called SHARP (SMRT/HDAC1 Associated Repressor Protein). SHARP is biologically important for the regulation of developmental and signaling pathways. However, its role at the molecular level is not well understood.[49] It is expressed in high levels in the brain, testis, spleen, and thymus and lower levels in kidney, liver, and spleen.[50] It has three RNA recognition motifs (RRMs) located near the N-terminus, which would suggest an RNA or DNA binding role.[49] The structure of SHARP provides some possible insight to its role at the molecular level, however, apart from its biological importance in regulating developmental and signaling pathways, little is known about this gene.

SHARP binds to co-repressor proteins silencing mediator for retinoid and thyroid (SMRT) hormone receptors and nuclear co-receptor repressor (NCOR). The SPOC domain of SHARP is sufficient for interaction with the C-terminal LSD peptide of SMRT and NCOR. In a β-galactosidase assay in yeast, SMRT LSD and N-CoR LSD interacted strongly with SHARP. Mammalian and yeast two hybrid assays were done to further investigate the interaction between SMRT and SHARP. SHARP was pulled out repeatedly using SMRT as bait.[49, 50] SHARP also recruits histone deacetylase activity (HDACs). HDACs remove acetyl groups causing chromatin to condense, which prevents transcription.[50] SHARP represses both liganded and non-liganded nuclear receptor

transcription by association with co-repressors SMRT and NCOR and HDACs.[37] PPAR-δ associates strongly with SMRT but only weakly with NCOR in a two-hybrid assay where the co-repressors served as bait. PPAR-δ was found to interact with SHARPs C-terminal receptor interaction domain, SPOC, by a coimmunoprecipitation assay. PPAR-δ also associates strongly with HDACs.[37] SHARP is directly associated with co-repressors SMRT and NCOR as well as interacting with PPAR-δ indirectly and directly. Regardless of these findings much more research is needed to truly understand the molecular function of SHARP.

I.9 Objectives of my study

The purpose of the current study is to confirm data obtained from a preliminary study of 19 participants of GOLDN. The gene expression of Bcl-6, which is hypothesized to contribute to the anti-inflammatory effects of fenofibrate, was measured in 29 individuals. The gene expression levels of Bcl-6 before and after treatment with fenofibrate were compared in these individuals. This study investigates the molecular mechanism of the anti-inflammatory effect of fenofibrate which is currently unknown. Inflammation plays a key role in CVD and having a greater understanding of how to treat inflammation would greatly benefit individuals suffering from CVD or those who are predisposed to this disease. Furthermore, determining the anti-inflammatory mechanism of the drug fenofibrate and other medications for treatment and prevention of CVD goes beyond CVD as chronic inflammation is present in many diseases and understanding the mechanisms of these medications could also benefit additional populations besides those affected by CVD.

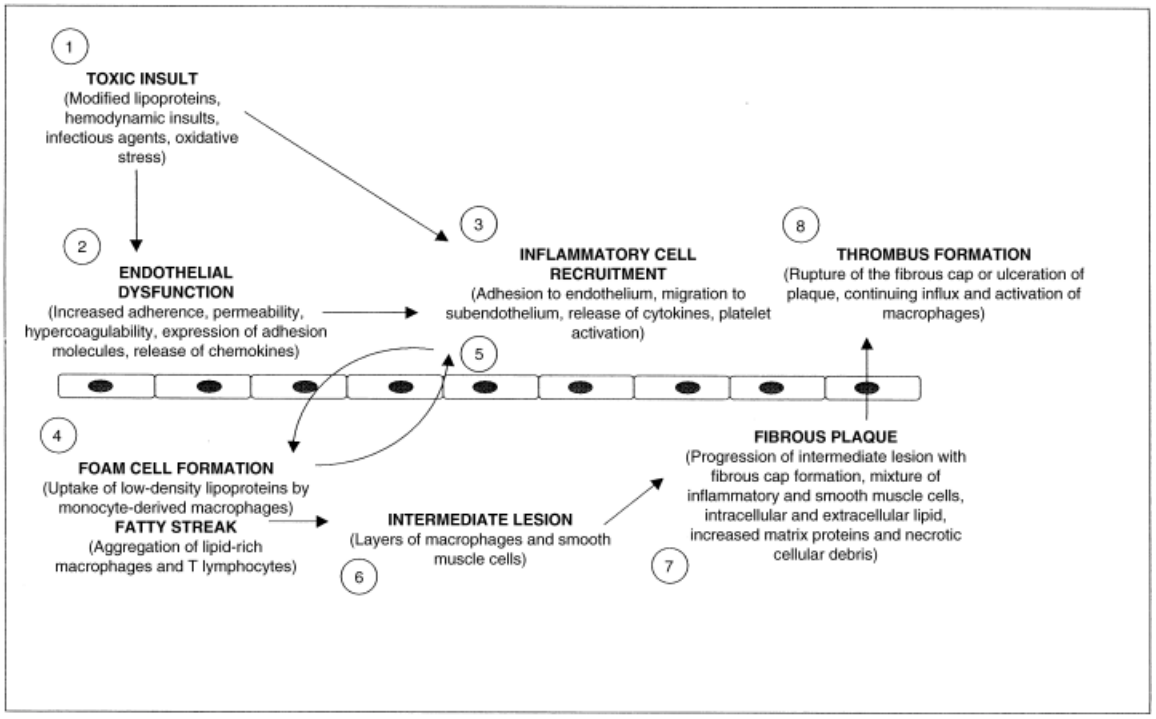

Figure I-1. The earliest changes that precede the formation of atherosclerosis take place in the endothelium (endothelial dysfunction), which is the result of exposure to various toxic insults. The endothelial cells increase expression of adhesion molecules and secrets various chemokines and growth factors. The increased adherence of monocyte/macrophages and T-cells precede their subendothelial migration. Subendothelial macrophages become large foam cells after lipid accumulation. The fatty streak can then progress to an intermediate lesion and ultimately to a fibrous plaque as a result of continued inflammation. The fibrous plaques increase in size and, by projecting into the lumen, may impede the blood flow and incite further thrombogenic stimuli. Reprinted from Arici et. al.[51]

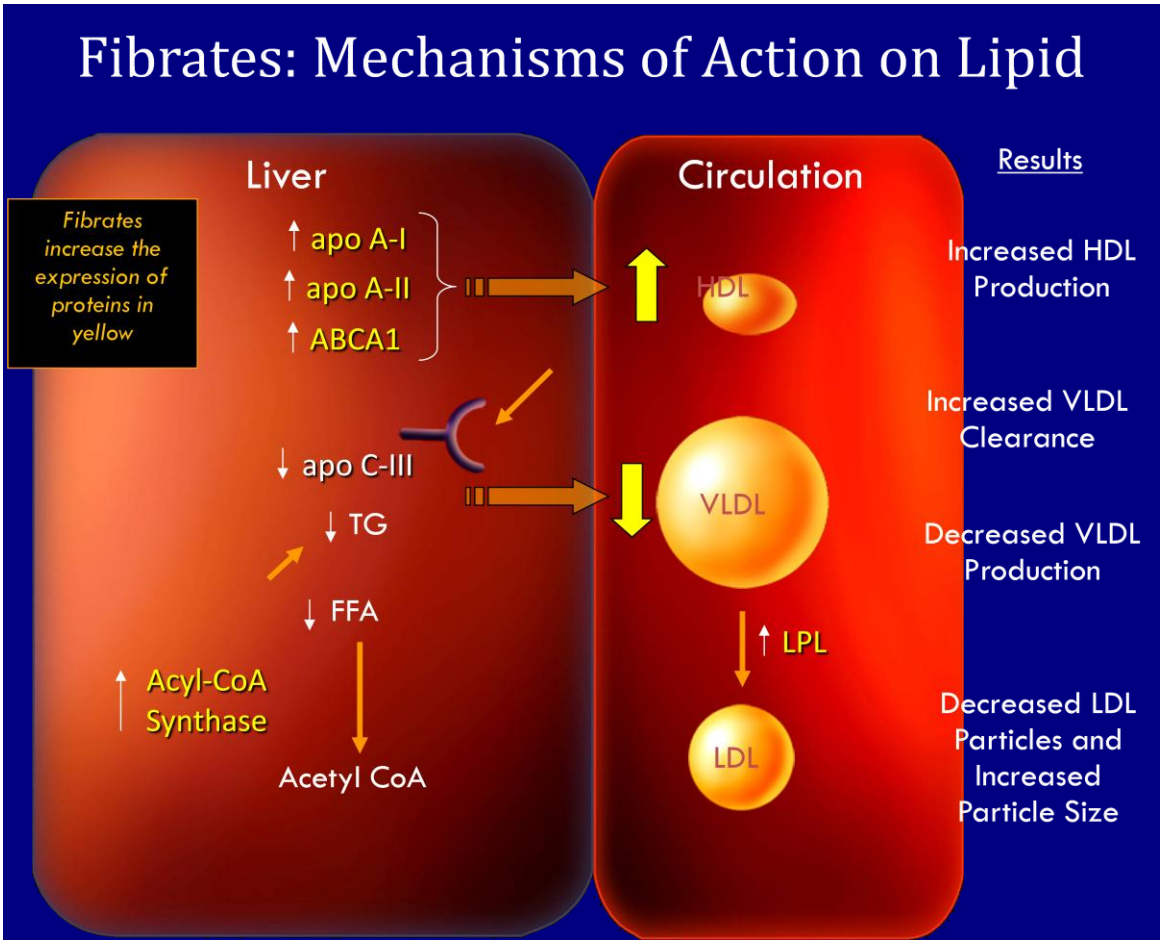

Figure I-2. The molecular mechanism of fibrates action on lipids.

MCP-1 inhibition

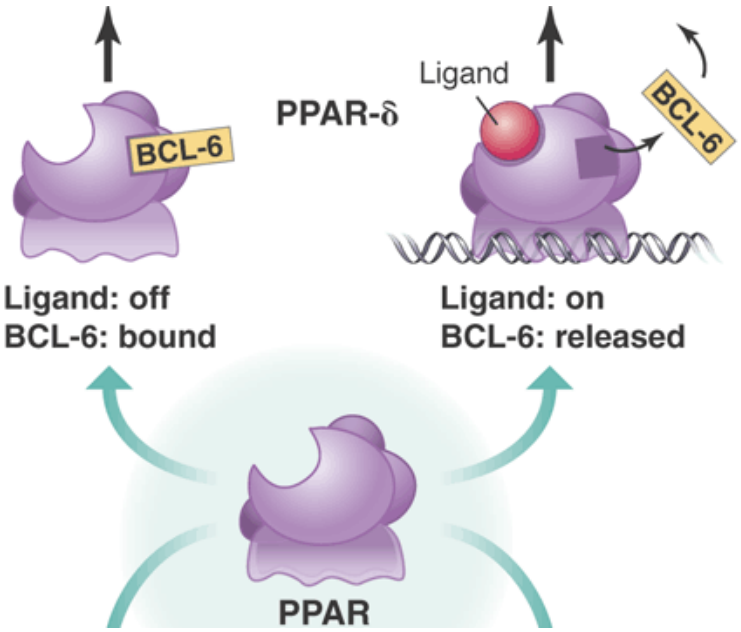

Figure I-3. A model for the involvement of Bcl-6 in PPAR-δ regulated MCP-1 inhibition. Unliganded PPAR-δ sequests BCL-6 protein. The ligand binding induces release of BCL-6 from PPAR-δ and results in suppression of MCP-1 gene transcription. Adapted from Plutzky et. al. [47]

II. MATERIALS AND METHODS

II.1 Study subjects

Participants were recruited through the Impact of of Genetic Determinants of Fenofibrate's Pharmacokinetics on Lipid Response (FPI) Study. The FPI study is approved by the Research Subjects Protection Program at the University of Minnesota (IRB# 078M15441, state clinical trial # NTC00613613). Potential participants' lipid profiles were assayed and screened for inflammatory markers VCAM and CRP. These individuals included 17 women and 17 men with a median age of 28.5 years (range, 20 to 73) [Table II-1]. Ethnic group was classified according to self-reported answers to the questions about race.

II.2 Study design

Normal

There are two phases in the FPI Study. Phase I potential subjects are screened to determine if they meet the requirements for the second phase of the study. A fasting blood sample was obtained from all participants for measurement of VCAM, hsCRP, and lipid profile (cholesterol,trigcylerides, HDL, LDL). The study defined elevated VCAM as greater than 600 ng/mL, and elevated hsCRP as greater than 1.2 mg/L. If an individual had elevated VCAM and hsCRP values, they were invited to participate in the second phase of the study. Two hundred and eight four subjects were screened in Phase 1, of the 284 subjects 34 qualified to participate in the second phase of the study.

Phase 2 subjects were dispensed fenofibrate (145 mg/ daily) for four weeks. The subjects had fasting blood samples drawn before and after treatment with fenofibrate [Figure II-1]. Five individuals who passed the screening phase, but no longer showed

elevated inflammatory markers at the beginning of Phase 2 were dropped from the study [Figure II-2].

Extended

Participants in the extended study continued their treatment with fenofibrate for an additional 4 weeks, totaling 8 weeks of fenofibrate treatment. Ten subjects were successfully recruited into the extended study. During this period, blood samples were drawn at 6 weeks and 8 weeks for a total of four blood draws.

II.3 RNA Extraction

Study subjects' total RNA was extracted from EDTA-anticoagulated blood according to the manufacter's instructors using the PerfectPure RNA Blood Kit 10 ml (5 Prime, Gaithersburg, MD). Extraction of RNA involves several steps including cell lysis, removal of proteins and genomic DNA, and purification of the ribonucleic acids. Precautions were taken to minimize degradation of RNA during the extraction.

II.4 Reverse Transcription

Reverse Transcription reactions were carried out in a 50 uL volume containing 250 ng of total RNA, 500 uM deoxynucleotide triphosphates mix, 5.5 mM $MgCl_2$, 2.5 uM Oligo dT's, 0.4 U/uL RNase inhibitor, 3.125 U/uL MultiScribe Reverse Transcriptase, and 1X RT Buffer (Applied Biosystems, Foster City, CA). After incubation at 25˚ C for 10 min, the temperature was increased to 37˚ C for 60 min, followed by reverse transcriptase inactivation at 95˚ C for 5 min using a GeneAmp PCR System 9600 (Perkin Elmer, Cor., Norwalk, CT).

II.5 Real Time PCR

II.5.1 18S

PCR reactions were carried out in a 20 uL volume containing 25 ng of cDNA, 1X SYBR Green PCR reaction mix (Applied Biosystems, Foster City, CA), and 450 nM of forward and reverse primer (IDT, Coralville, IA) [Table II-1]. After UNG activation at 50˚ C for 2 min, AmpliTaq was activated for 10 min at 95 C, followed by the temperature being cycled 40 times (at 95˚ C for 15 sec for denaturation, and annealing/extension temperature of 60˚ C for 1 min), after each PCR reaction cycle, the melting curve was measured (at 95˚ C for 15 sec, 60˚ C for 1 min, and at 95˚ C for 15 sec),using a 7500 Fast Real-Time PCR machine **(**Applied Biosystems, Foster City, CA)**.**

II.5.2 Bcl-6 & Spen

PCR reactions were carried out in a 20 uL volume containing 25 ng of cDNA, 1X SYBR Green PCR reaction mix Applied Biosystems, Foster City, CA), and 900 nM forward and reverse primer (IDT, Coralville, IA) [Table II-1]. After UNG activation at 50˚ C for 2 min, AmpliTaq was activated for 10 min at 95 C, followed by the temperature being cycled 40 times (at 95˚ C for 15 sec for denaturation, and annealing/extension temperature of 60˚ C for 1 min), after each PCR reaction cycle, the melting curve was measured (at 95˚ C for 15 sec, 60˚ C for 1 min, and at 95˚ C for 15 sec),using a 7500 Fast Real-Time PCR machine **(**Applied Biosystems, Foster City, CA)**.**

II.6 Inflammatory markers and lipids

Lipid profiles (cholesterol, triglycerides, HDL-C, LDL-C) and hsCRP measurements were performed in the Collaborative Studies Clinical Laboratory of Univ of MN. Fairview.

II.6.1 Cholesterol

Total cholesterol is measured in EDTA plasma using a cholesterol oxidase method (Roche Diagnostics, Indianapolis, IN 46250) on a Roche Modular P Chemistry Analyzer (Roche Diagnostics Corporation). This method incorporates cholesterol esterase and peroxidase in the reagent and monitors cholesterol oxidation at 500 nm upon conversion of 4-aminoantipyrine to quinoneimine. This enzymatic method is standardized with a serum standard prepared in our laboratory and frozen at -70°C. The assigned value of this standard is traceable to replicate Abell-Kendall cholesterol analysis performed by a CDC/NHLBI Cholesterol Reference Method Laboratory Network laboratory. The calibration of this assay is regularly monitored by the CDC/NHLBI Lipid Standardization Program. The NCEP program recommends reference range of <200 mg/dL. The laboratory CV is 1.6%.

II.6.2 Triglyceride

Triglyceride is measured in EDTA plasma using Triglyceride GB reagent (Roche Diagnostics, Indianapolis, IN 46250) on a Roche Modular P Chemistry Analyzer. (Roche Diagnostics Corporation). This assay performs an automated glycerol blank by taking a spectrophotometric reading after endogenous glycerol has reacted and before lipase is added to release the glycerol from the triglyceride. This method is calibrated with a frozen serum standard prepared in our laboratory and frozen at -70°C. We have assigned this calibrator by comparison to CDC reference materials. The NCEP program recommends reference range of <150 mg/dL. The laboratory CV is 4.0%.

II.6.3 HDL-Cholesterol

HDL-cholesterol is measured in EDTA plasma using the cholesterol oxidase cholesterol method (Roche Diagnostics) after precipitation of non-HDL-cholesterol with

magnesium/dextran. This method is standardized as described for the cholesterol assay; and calibration of the assay is regularly monitored by the CDC/NHLBI Lipid Standardization Program. The NCEP program recommends reference range of >40 mg/dL. The laboratory CV is 2.9%.

II.6.4 LDL-Cholesterol Calculated

LDL-cholesterol is calculated in plasma specimens having a triglyceride value <400 mg/dL using the formula of Friedewald et al. (Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. (Clin Chem. 1972;18:499-502). The NCEP program recommends reference range of <100 mg/dL.

II.6.5 C-reactive Protein, High Sensitive (hsCRP)

hsCRP is measured in serum or plasma using a latex-particle enhanced immunoturbidimetric assay kit (Roche Diagnostics, Indianapolis, IN 46250) and read on the Roche Modular P Chemistry analyzer (Roche Diagnostics). The reference range is 0 – 5 mg/L. The inter-assay CV in our laboratory is 4.5%.

II.6.6 (soluble) Vascular Cell Adhesion Molecule-1 (sVCAM-1)

sVCAM-1 (soluble vascular cell adhesion molecule-1) is measured using the quantitative sandwich enzyme technique of the enzyme immunoassay Human sVCAM-1 Quantikine assay from R & D Systems (Minneapolis, MN). The intensity of the color is measured on a SpectraMax spectrophotometer (Molecular Devices, Sunnyvale, California). The inter-assay CV range reported in the kit insert is 8.9-10.2% for the serum or plasma assay. Our laboratory CV is <10%.

II.7 Gene expression analysis

Gene expression analysis was done with 7500 Software v2.0 on the 7500 Fast-PCR machine. (Applied Biosystems, Foster City, CA). The threshold value was set at 0.200000 for all plates. Triplicate Ct values of subjects had to be within 0.5 Ct of each other. If an outlier was present, the two remaining Ct values had to be within 0.1 Ct of each other. Relative change in gene expression (n-fold) was calculated according to Plaff et. al.[52]

II.8 Statistical analysis

Wilcoxon signed rank test was performed to compare the relative gene expression; sVCAM-1, hsCRP, and lipid profile expression before and after fenofibrate treatment. A P-value <0.05 was considered to be statistically significant.

Table II.1 Table of study subjects

Subject ID	Age Screening (Years)	Gender Male=1 female=2	BMI
1	22	1	28.90
2	30	2	41.45
3	21	2	21.02
4	24	1	22.72
5	20	1	25.61
6	26	1	26.76
7	28	1	34.44
8	30	2	26.72
9	32	2	30.04
10	18	1	22.27
11	25	2	23.32
12	20	1	22.86
13	30	1	35.59
14	26	2	24.94
15	48	2	32.41
16	24	1	33.50
17	28	2	21.49
18	31	2	33.45
19	58	1	29.67
20	22	1	45.69
21	30	2	39.43
22	57	1	30.30
23	24	2	21.53
24	18	2	28.21
25	44	1	21.36
26	43	2	24.82
27	19	1	24.16
28	71	2	24.72
29	60	1	35.82
30	20	1	21.25
31	21	1	21.80
32	25	2	23.26
33	27	2	37.69
34	21	2	22.22

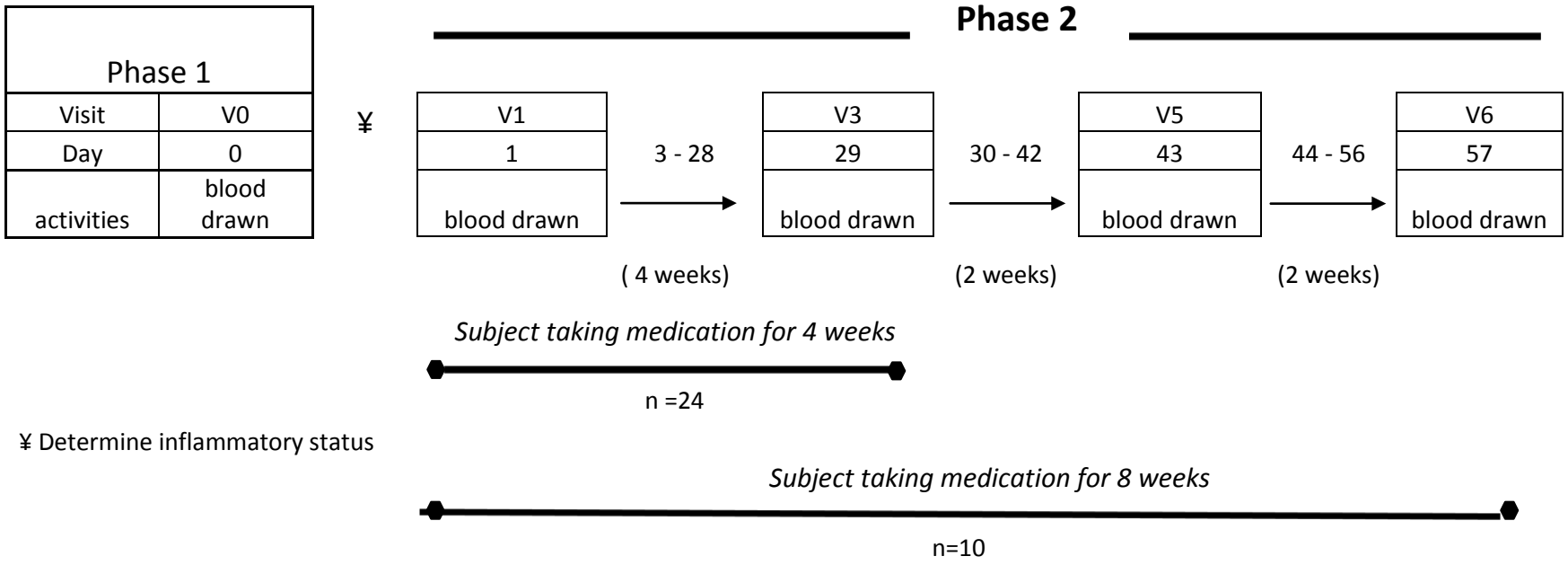

Figure II-1 Study design diagram

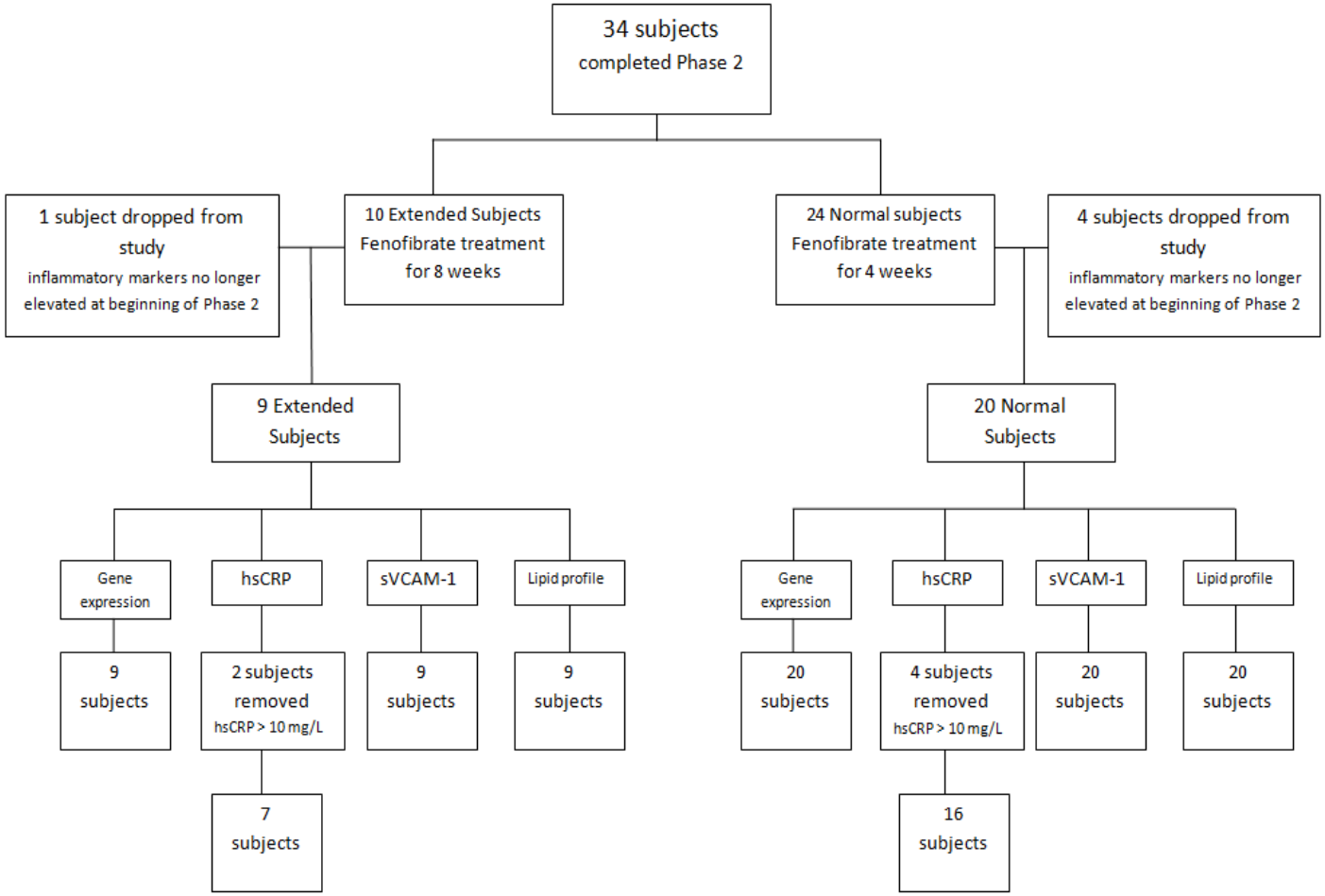

Figure II-2 Flowchart of study subjects

Table II-2 PCR primer sequences

Gene	Primer sequence (5'-3')	Length
Bcl-6	**F: CCTCACGGTGCCTTTTTTCA**	**201 bp**
	R:GTAATGCAGTTTAGACACAGCCAAA	
Spen	**F:GTGGTGCTGCTACCTTGTATGTTT**	**213 bp**
	R:CAGAGTGTCACAGCCTGTCTTCA	
18S	**F:GGCGGCTTTGGTGACTCTAG**	**180 bp**
	R:CCTGCTGCCTTCCTTGGAT	

III RESULTS

III.1 Comparison of hsCRP before and after fenofibrate treatment

III.1.1 hsCRP measurement – 4 weeks of treatment

III.1.1.1 All subjects

For hsCRP measurement of subjects treated for 4 weeks with fenofibrate, 29 pretreatment and 29 post treatment samples were measured. Due to hsCRP levels greater than 10 mg/L, which is indicative of recent infection or trauma six subjects were removed as outliers. The average baseline value of hsCRP was 2.8 ± 0.4 mg/L , and 1.8 ± 0.2 mg/L after treatment [Table III-1]. The difference is not statistically significant ($p < 0.70$) [Figure III-1]. Individuals were based into two groups based on their baseline hsCRP values. The second tile showed a significant decrease after fenofibrate treatment ($p < 0.01$) [Figure III-1A].

III.1.1.2 Individuals with elevated hsCRP and sVCAM-1

For hsCRP measurement of subjects treated for four weeks with fenofibrate, 17 pretreatment and 17 post treatment samples were measured [Figure III-2]. Due to hsCRP levels greater than 10 mg/L, which is indicative of recent infection or trauma 5 subjects were removed as outliers. The average baseline value of hsCRP was 3.5 ± 0.7 mg/L , and 2.0 ± 0.4 mg/L after treatment [Table III-2]. The difference is not statistically significant ($p < 0.30$) [Figure III-2A].

III.1.1.3 Individuals with elevated hsCRP or sVCAM-1

For hsCRP measurement of subjects treated for 4 weeks with fenofibrate, 12 pretreatment and 12 post treatment samples were measured [Figure III-2]. Due to hsCRP levels greater than 10 mg/L, which is indicative of recent infection or trauma 1 subject

was removed as outliers. The average baseline value of hsCRP was 2.1 ± 0.5 mg/L, and 1.5 ± 0.3 mg/L after treatment [Table III-2]. The difference is not statistically significant ($p < 0.20$) [Figure III-3].

III 1.2 hsCRP measurement- 8 weeks of treatment

For hsCRP measurement of subjects treated for 8 weeks with fenofibrate, 9 pretreatment and 9 post treatment samples were measured. Due to hsCRP levels greater than 10 mg/L, which is indicative of recent infection or trauma 2 subjects were removed as outliers. The average baseline value of hsCRP was 2.9 ± 0.8 mg/L, while 2.3 ± 0.5 mg/L in 4 weeks of treatment, 2.4 ±0.6 mg/L after 6 weeks of treatment, and 3.0 ±0.9 mg/L after treatment for 56 8 weeks [Table III-3]. There were no significant differences between the pretreatment and post treatment of 4 weeks, 6 weeks, or 8 weeks ($p < 0.81$, $p < 0.21$, $p < 0.93$, respectively) [Figure III-4].

III.2 Comparison of sVCAM-1 before and after fenofibrate treatment

III 2.1 sVCAM-1 measurement – 4 weeks of treatment

III.2.1.1 All subjects

For sVCAM-1 measurement of subjects treated for 4 weeks with fenofibrate, 29 pretreatment and 29 post treatment samples were measured. The average baseline value of sVCAM-1 was 704.6 ± 51.7 ng/ml, and 667.7 ± 34.2 ng/ml after four weeks of treatment. The difference is not statistically significant ($p < 0.15$) [Table III-1][Figure III-5]. Individuals were grouped into quartiles based on their baseline sVCAM-1 values. Quartiles three and four are significantly decreased after 4 weeks of fenofibrate treatment ($p < 0.04$, $p<0.01$). Quartiles one and two were not significantly decreased ($p < 0.54$, $p < 0.21$) [Figure III-5A].

III.2.1.2 Individuals with elevated hsCRP and sVCAM-1

For sVCAM-1 measurement of subjects treated for 4 weeks with fenofibrate, 17 pretreatment and 17 post treatment samples were measured. The average baseline value of sVCAM-1 was 794.7 ± 79.0 ng/ml, and 734.3 ± 51.5 ng/ml after 4 weeks of treatment [Table III-2]. The difference is not statistically significant ($p < 0.15$) [Figure III-6].

III.2.1.3 Individuals with elevated hsCRP or sVCAM-1

For sVCAM-1 measurement of subjects treated for 4 weeks with fenofibrate, 12 pretreatment and 12 post treatment samples were measured. The average baseline value of sVCAM-1 was 576.9 ± 31.6 ng/ml, and 573.8 ± 24.7 ng/ml after 4 weeks of treatment [Table III-2]. The difference is not statistically significant ($p < 0.90$) [Figure III-7].

III 2.2 sVCAM-1 measurement- 8weeks of treatment

For sVCAM-1 measurement of subjects treated for 8 weeks with fenofibrate, 9 pretreatment and 9 post treatment samples were measured. The average baseline value of sVCAM-1 was 763.9 ± 154.4 ng/ml, while 734.4 ± 93.2 ng/ml in 4 weeks of treatment, 789.1 ± 116.1 ng/ml after 6 weeks of treatment, and 785.5 ± 111.6 ng/ml after treatment for 8 weeks [Table III-3]. There were no significant differences between the pretreatment and post treatment of 4 weeks, 6 weeks, or 8 weeks ($p < 1$, $p < 0.65$, $p < 0.35$, respectively) [Figure III-8].

III.3 Comparison of lipid profiles before and after fenofibrate treatment

III.3.1 Lipid profile- 4 weeks of treatment

29 pretreatment and 29 post treatment samples were measured for cholesterol, triglycerides, LDL-C and HDL-C. The average baseline of cholesterol was 182.0 ± 6.0 mg/dL, and 158.7 ± 6.4 mg/dL after treatment. There was a significant difference

between the pretreatment and post treatment samples ($p < 0.00005$). The average baseline of triglycerides was 144.7 + 20.8 mg/dl, and 98.7 + 13.6 mg/dL after treatment. The difference was significant ($p < 0.00005$). There was also a significant difference for LDL-C (average baseline was 110.5 ± 5.4 mg/dL; post treatment was 94.0 ±5.1 mg/dL; $p<0.00002$). For HDL-C, the average baseline was 43.7 ± 2.1 mg/dL; post treatment it was 45.0 ± 2.0 mg/dL. The difference was not significant ($p<0.62$) [Table III-1][Figure III-9].

III. 3.2 Lipid profile – 8 weeks of treatment

Cholesterol, triglycerides, LDL-C, and HDL-C were measured in 9 pre and post treatment samples. Cholesterol measurements for week 0, week 4, week 6, week 8 are 207.9 ± 5.7 mg/dL, 190.1 ±10.5 mg/dL, 190.5 ± 7.3 mg/dL, and 188.5 ± 7.8 mg/dL, respectively. All differences between week 0 measurement and post treatment measurements (week 4, week 6, week 8) were significant ($p<0.02$,$p <0.003$, $p <0.02$ respectively). The baseline value of triglycerides is 167.8 ± 56.9 mg/dL and the post treatment measurements for week 4, week 6, and week 8 are 122.9 ± 32.4 mg/dL, 103.8 ± 21.0 mg/dL, and 108.1 ± 24.2 mg/dL. Differences between week 0 and post treatment measurements at week 6 and week 8 are significant ($p < 0.01$, $p < 0.003$, respectively). For LDL-C measurement at week 0 was 135.9 ± 7.0 mg/dL and post treatment values were 118.7 ± 8.0 mg/dL, 120.5 ± 7.7 mg/dL, 119.1 ± 6.8 mg/dL for week 4, week 6 and week 8 respectively. All differences were significant ($p < 0.003$, $p < 0.007$, $p < 0.05$,respectively). The average baseline value of HDL-C was 45.5 ± 4.9 mg/dL; post treatment it was 47.0 ± 4.2 mg/dL , 49.8 ± 4.5 mg/dL , 50.4 ± 5.1 mg/dL , for week 4, week 6 and week 8 respectively. There was significant difference between pretreatment

and post treatment samples at week 6 and week 8 ($p < 0.02$, $p < 0.05$) [Table III-3][Figure III-10].

III. 4 Comparison of gene expression before and after treatment

III. 4.1 Gene expression- 4 weeks of treatment

III.4.1.1 All subjects

Gene expression was measured before and after treatment of fenofibrate for genes Bcl-6 and Spen in all 29 subjects. The pretreatment gene expression fold change is normalized to 1. After 4 weeks of treatment the relative expression (n-fold) of Bcl-6 and Spen is 0.903 ± 0.09 and 0.845 ± 0.05, respectively [Table III-4]. The change in expression from pretreatment and post treatment is significant for Spen only ($p < 0.08$, $p < 0.003$,respectively) [Figure III-11].

III.4.1.2 Individuals with elevated hsCRP and sVCAM-1

Gene expression was measured before and after treatment of fenofibrate for genes Bcl-6 and Spen. The pretreatment gene expression fold change is normalized to 1. After 4 weeks of treatment the relative expression (n-fold) of Bcl-6 and Spen is 0.864 ± 0.12 and 0.928 ± 0.04, respectively [Table III-2]. The change in expression from pretreatment and post treatment is significant for Bcl-6 ($p < 0.03$, $p < 0.14$, respectively) [Figure III-12].

III.4.1.3 Individuals with elevated hsCRP or sVCAM-1

Gene expression was measured before and after treatment of fenofibrate for genes Bcl-6 and Spen. The pretreatment gene expression fold change is normalized to 1. After 4 weeks of treatment the relative expression (n-fold) of Bcl-6 and Spen is 0.959 ± 0.14 and

0.727 ± 0.05, respectively [Table III-2]. The change in expression from pretreatment and post treatment is significant for Spen ($p < 0.73$, $p < 0.004$, respectively) [Figure III-13].

III.4.2 Gene expression- 8 weeks of treatment

Gene expression was measured before and after treatment of fenofibrate for genes Bcl-6 and Spen. The pretreatment gene expression fold change is normalized to 1. The relative expression (n-fold) of Bcl-6 after 4 weeks, 6 weeks, and 8 weeks is 0.817 ± 0.13, 0.757 ± 0.09, 0.921±0.11, respectively. There was no significant change in gene expression between pre and post treatment ($p < 0.12$, $p < 0.07$, $p < 0.25$, respectively). The relative expression (n-fold) of Spen after 4 weeks, 6 weeks, and 8 weeks is 0.866 ± 0.05, 1.047 ± 0.13, 0.964 ± 0.06, respectively [Table III-5]. The change in expression from pretreatment and post treatment is not significant ($p < 0.07$, $p < 0.73$, $p < 0.57$, respectively) [Figure III-14].

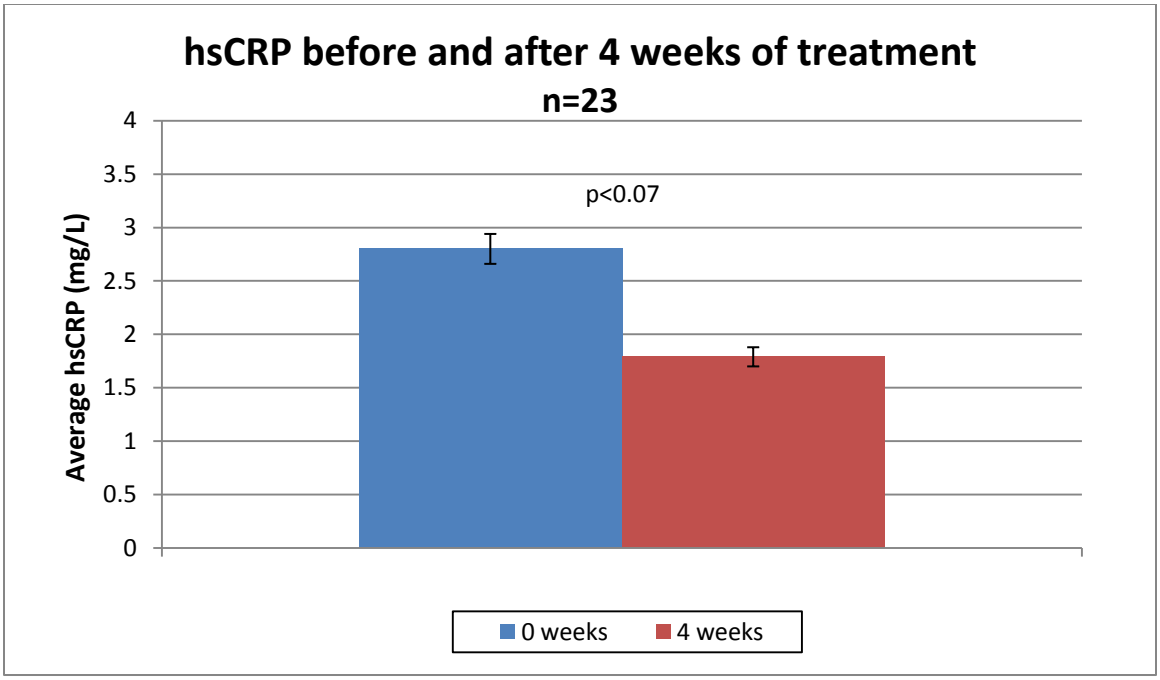

Figure III-1 hsCRP expression before and after 4 weeks of fenofibrate treatment

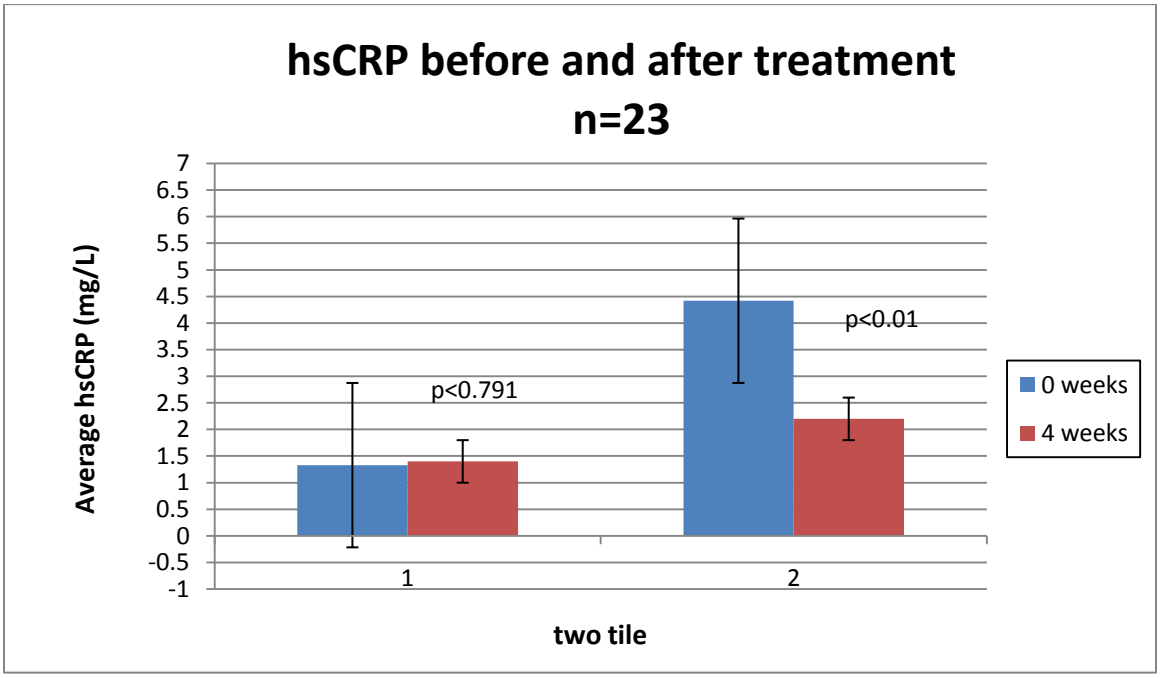

Figure III-1A Two tiles of hsCRP expression before and after 4 weeks of fenofibrate treatment

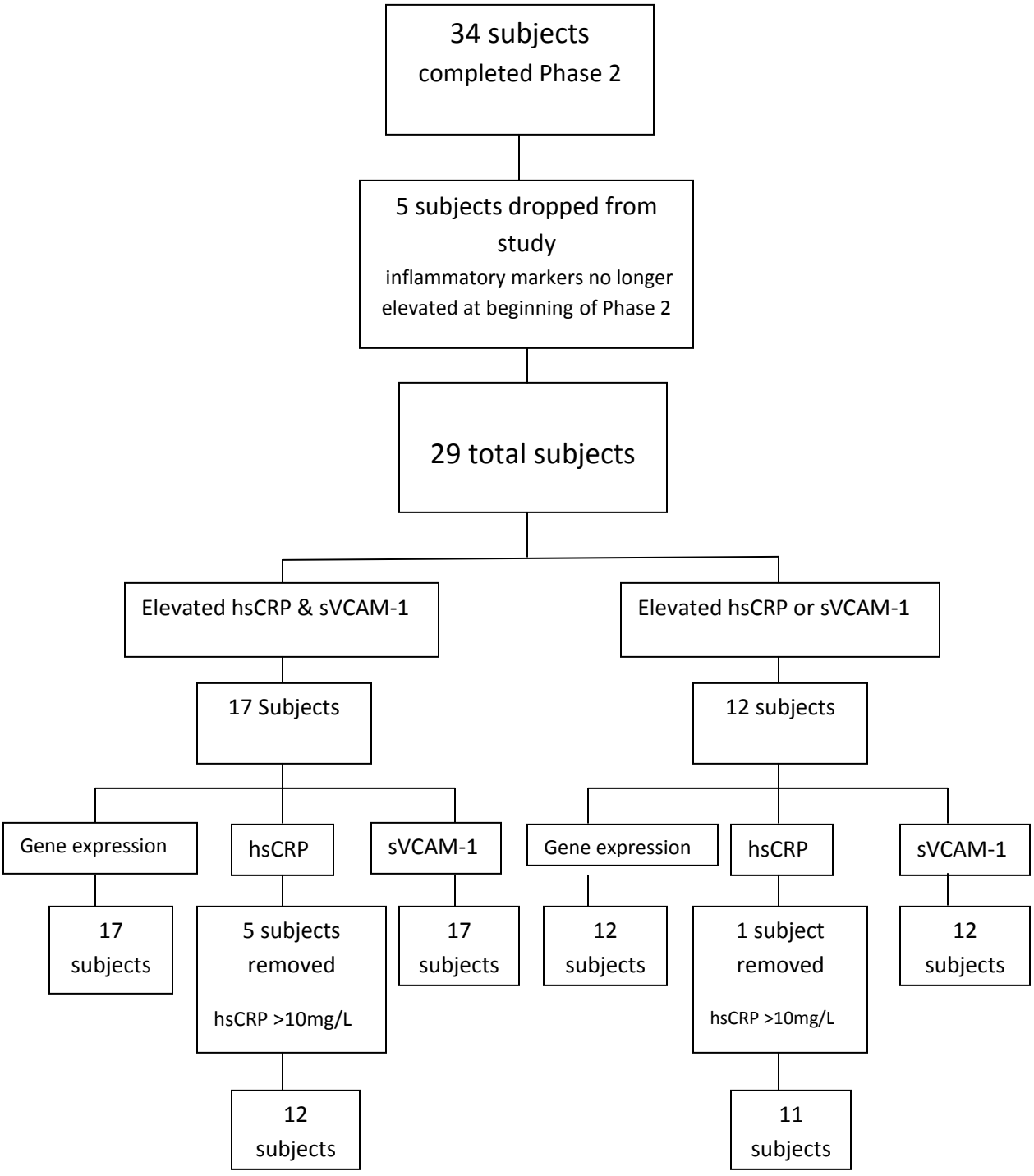

Figure III-2 Population dispartiy flowchart

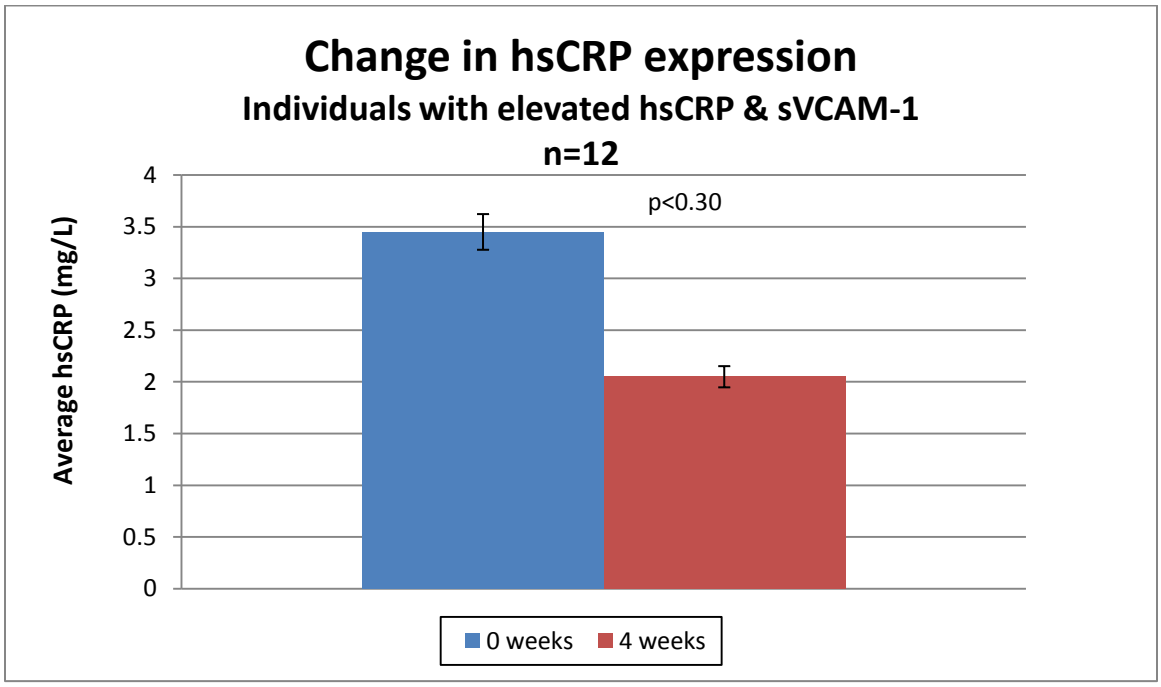

Figure III-2A hsCRP expression of individuals with elevated hsCRP & sVCAM-1 before and after 4 weeks of fenofibrate treatment

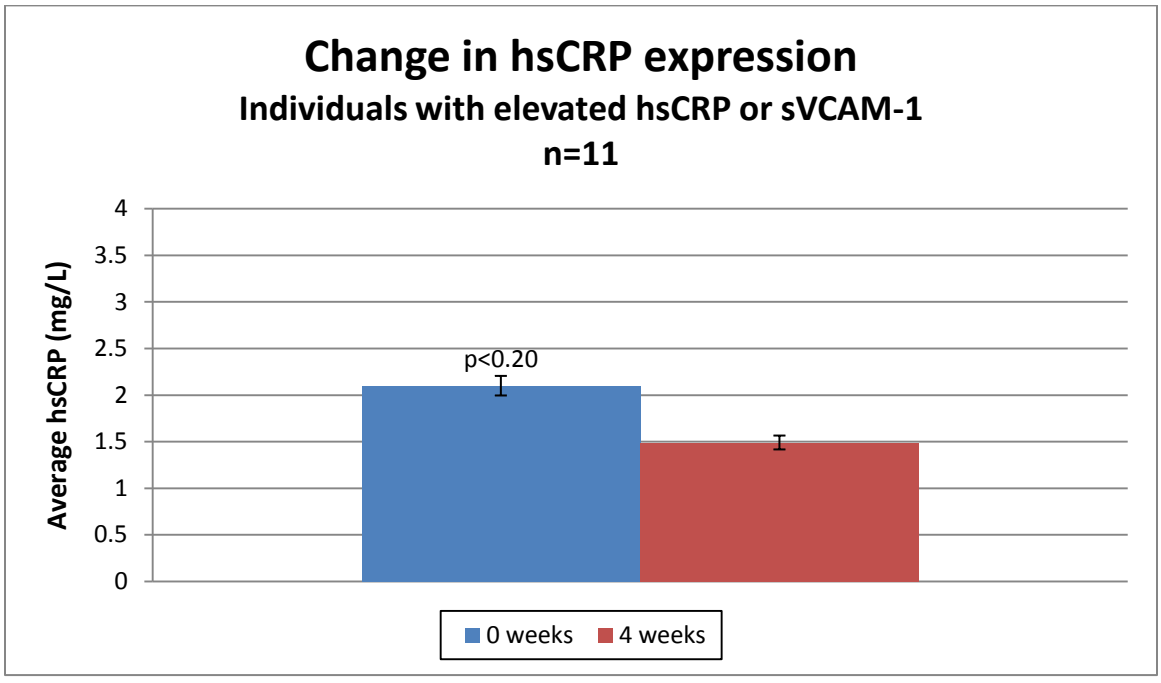

Figure III-3 hsCRP expression of individuals with elevated hsCRP or sVCAM-1 before and after 4 weeks of fenofibrate treatment

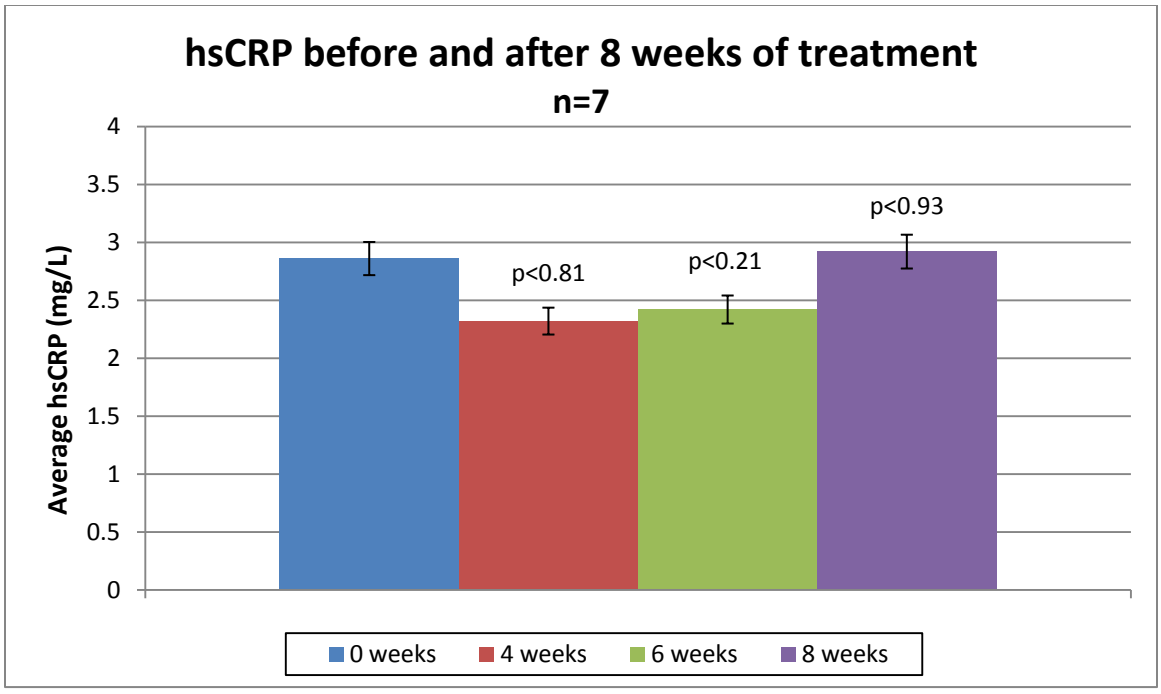

Figure III-4 hsCRP expression before and after eight weeks of fenofibrate treatment

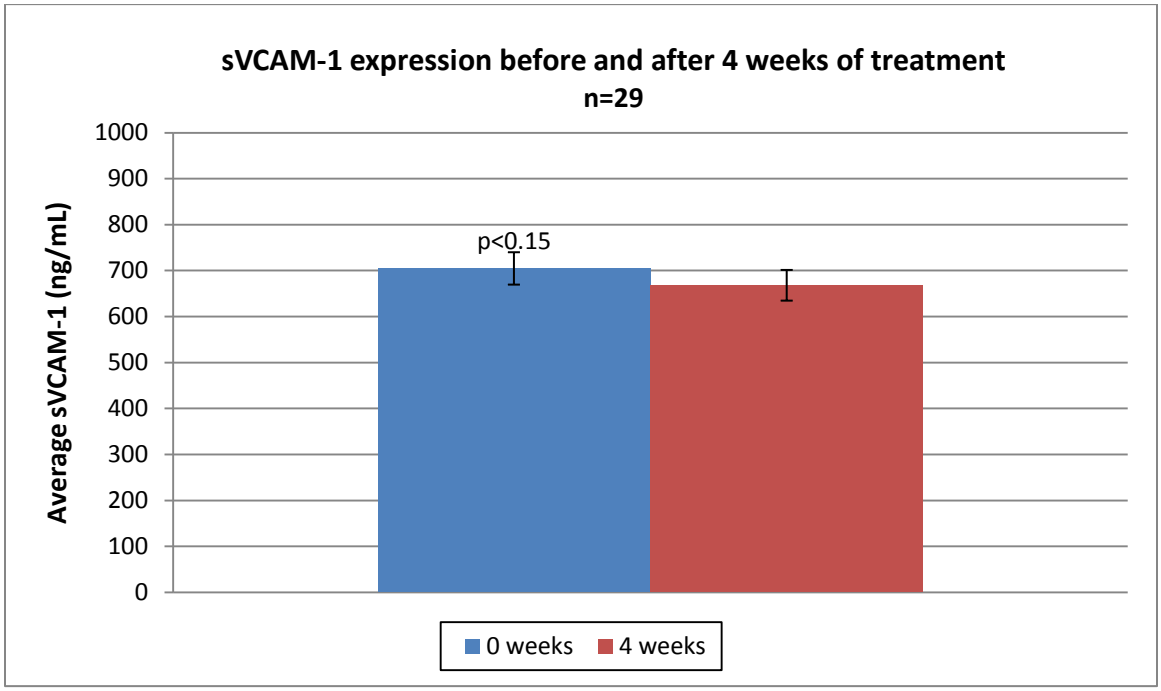

Figure III-5 sVCAM-1 expression before and after four weeks of fenofibrate treatment

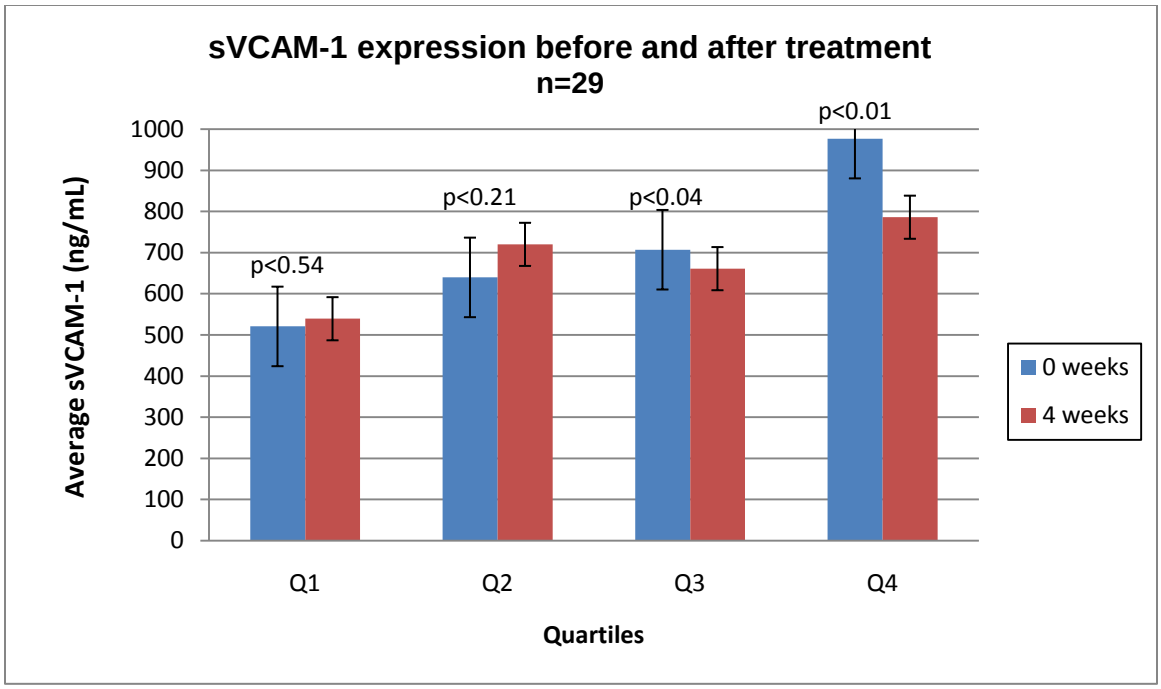

Figure III-5A Quartiles of sVCAM expression before and after four weeks of fenofibrate treatment

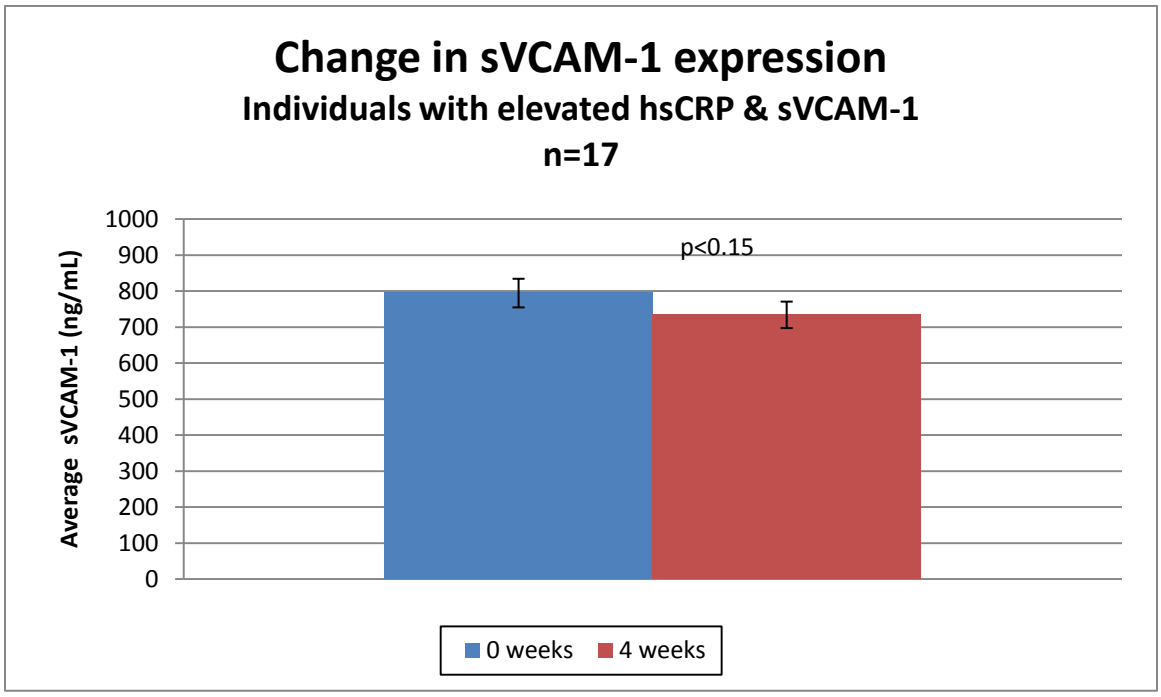

Figure III-6 sVCAM-1 expression in individuals with elevated hsCRP & sVCAM-1 before and after four weeks of fenofibrate treatment

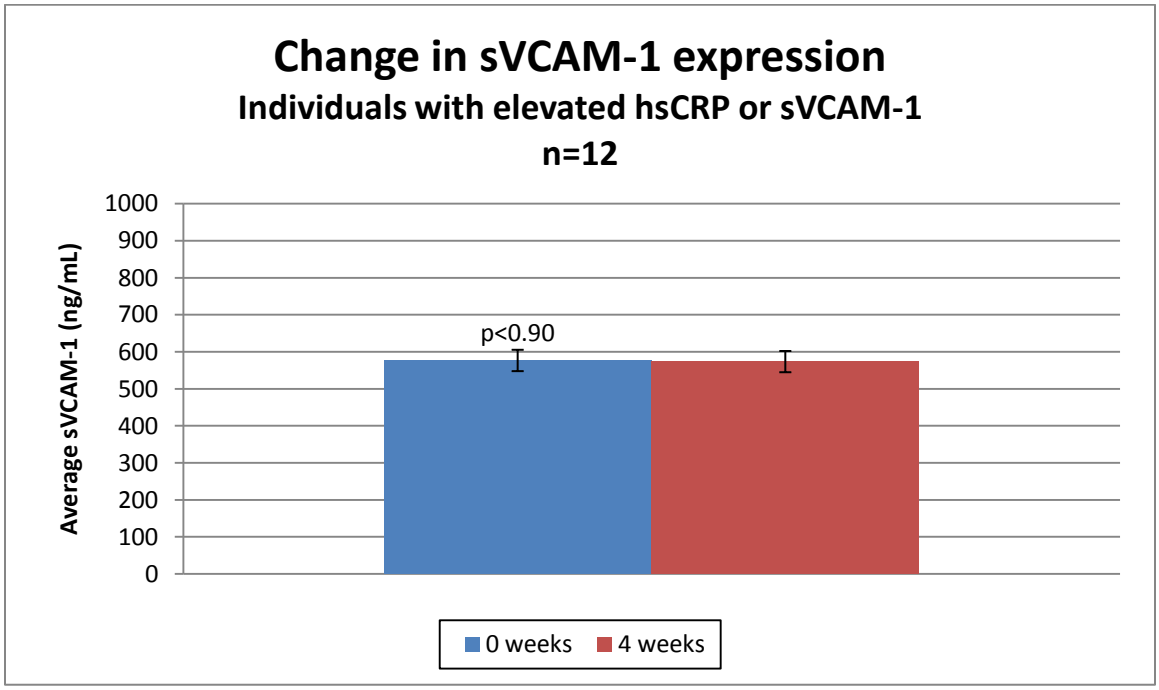

Figure III-7 sVCAM-1 expression in individuals with elevated hsCRP or sVCAM-1 in individuals before and after four weeks of fenofibrate treatment

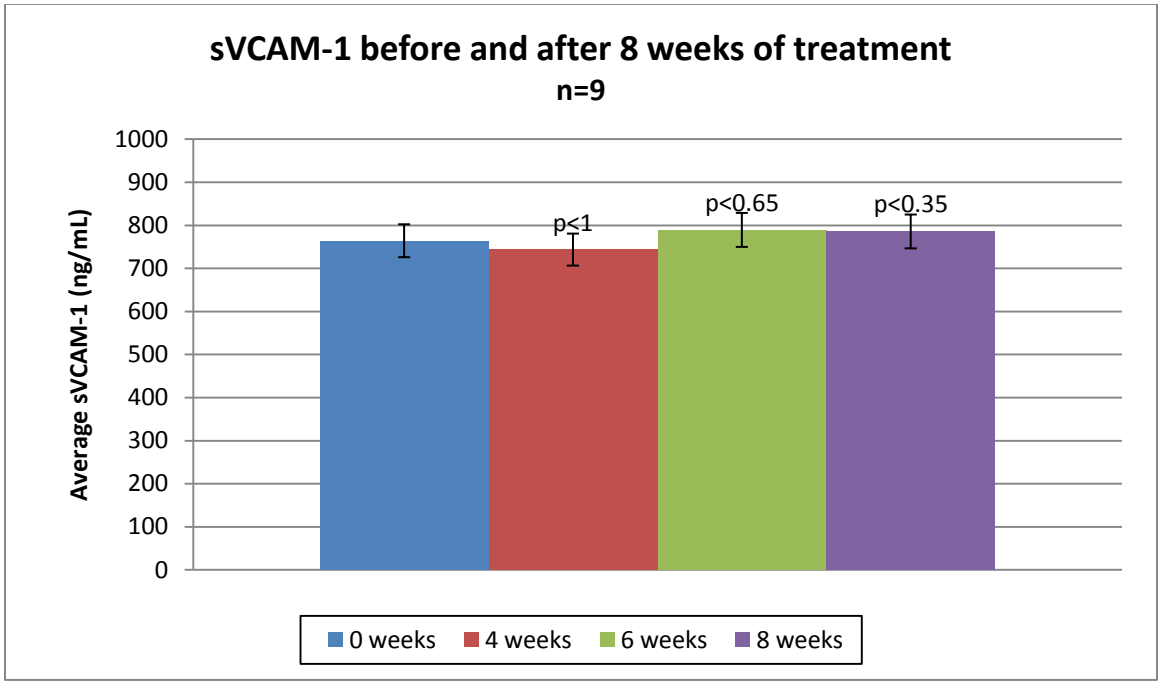

Figure III-8 sVCAM-1 expression before and after eight weeks of fenofibrate treatment

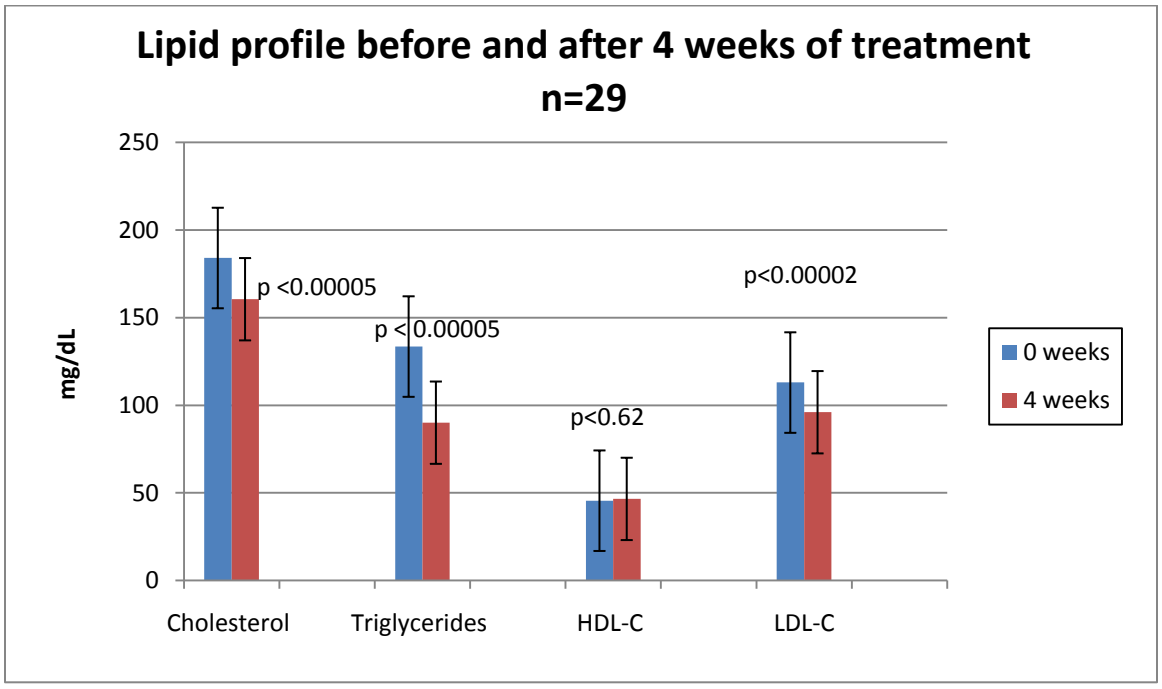

Figure III-9 Lipid profile before and after four weeks of fenofibrate treatment

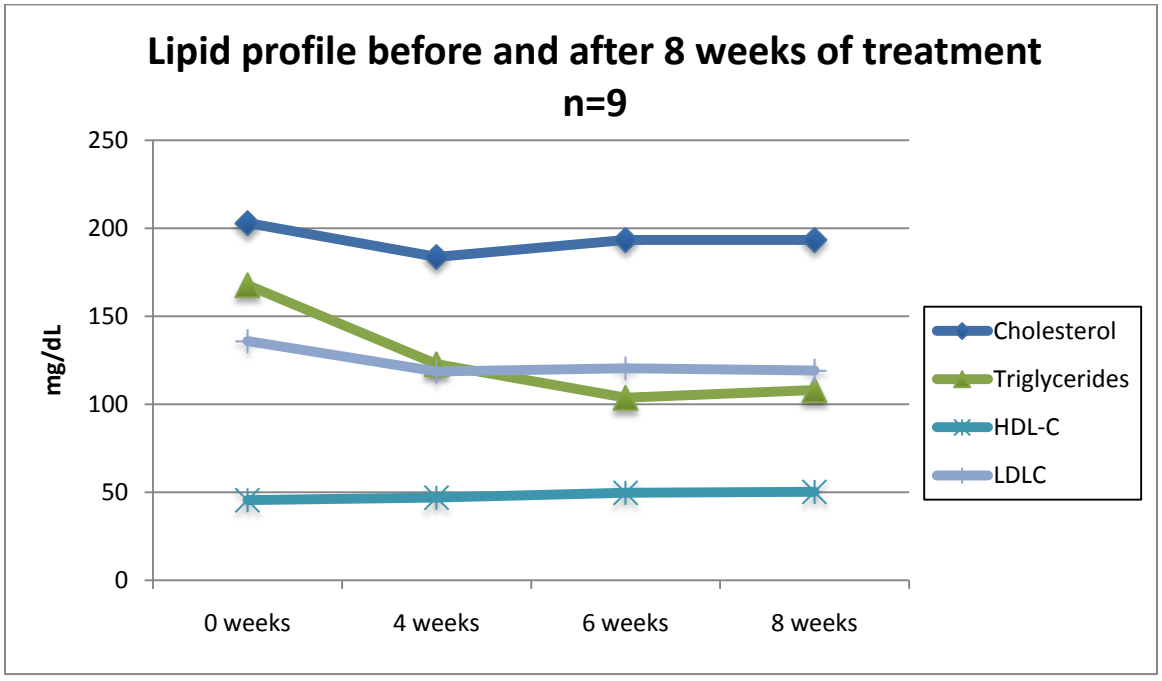

Figure III-10 Lipid profile before and after eight weeks of fenofibrate treatment

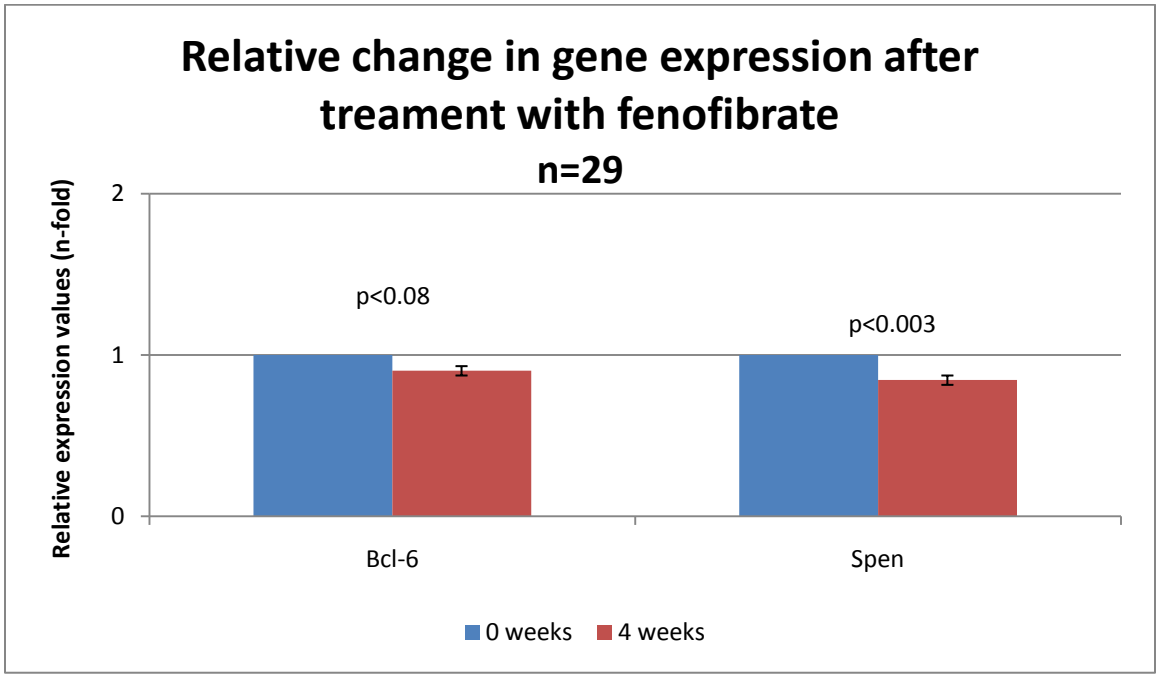

Figure III-11 Relative change in gene expression before and after four weeks of fenofibrate treatment

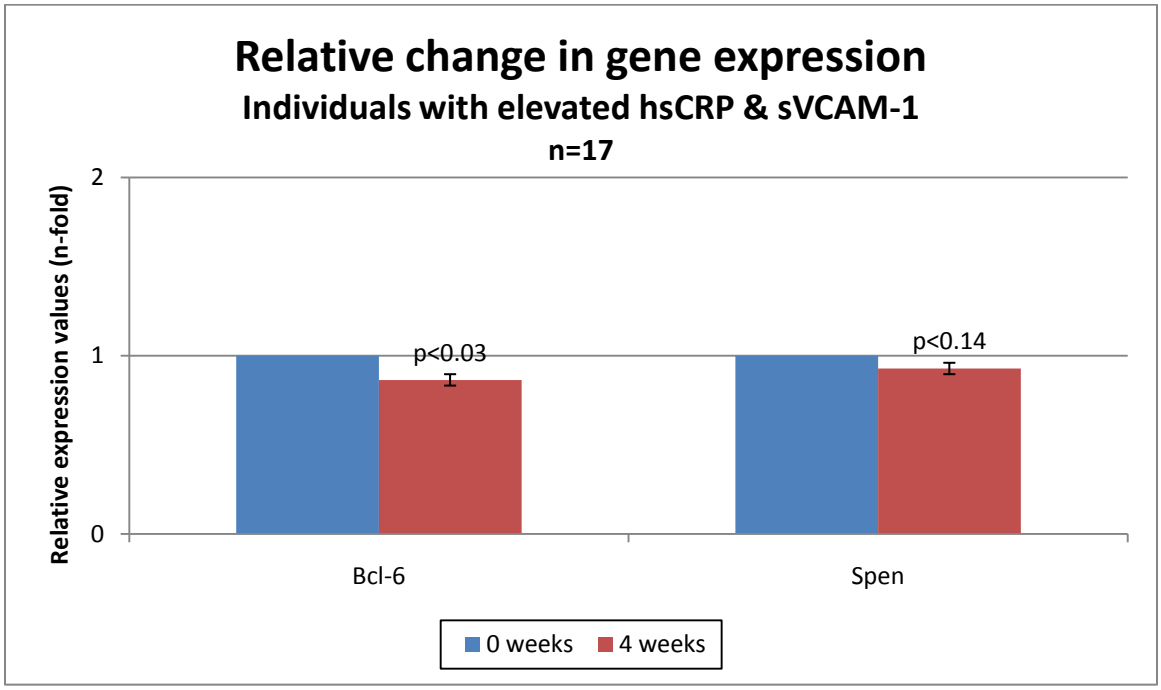

Figure III-12 Relative change in gene expression in individuals with elevated hsCRP & sVCAM-1 before and after four weeks of fenofibrate treatment

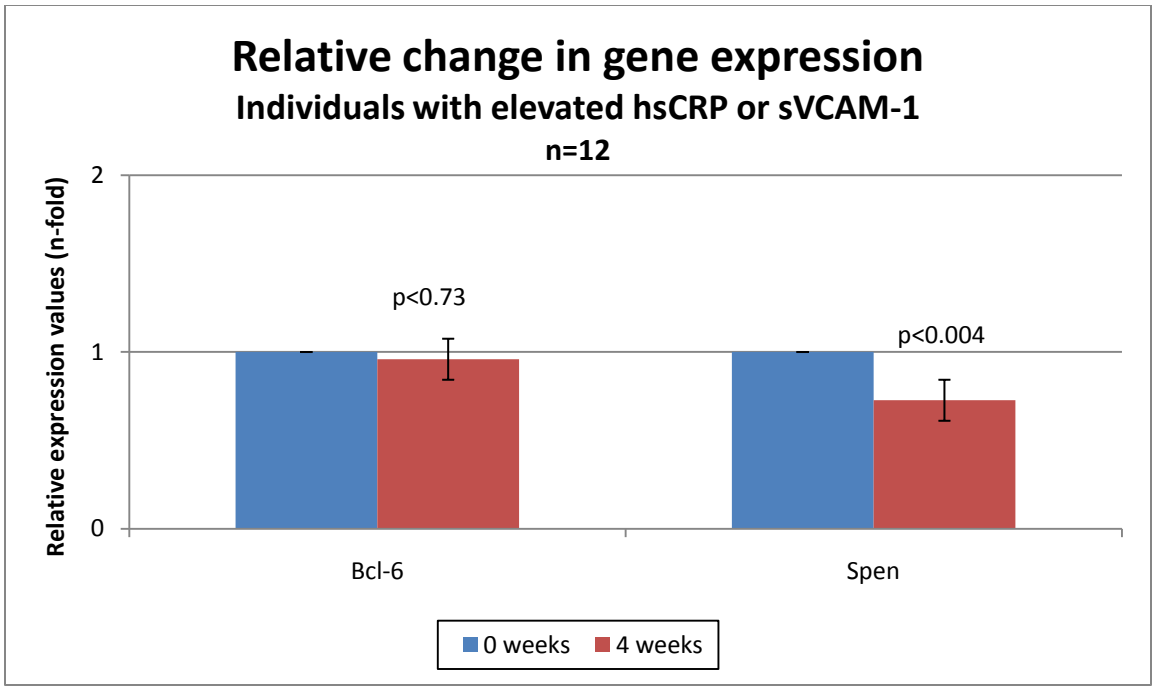

Figure III-13 Relative change in gene expression in individuals with elevated hsCRP or sVCAM-1 before and after four weeks of fenofibrate treatment

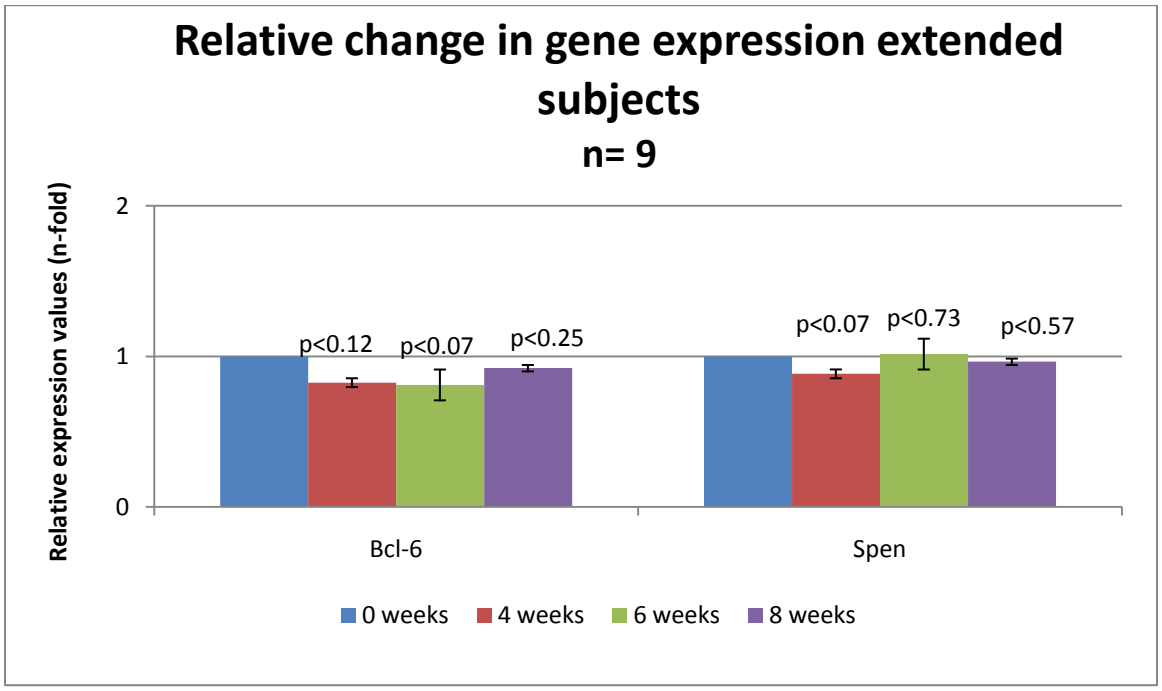

Figure III-14 Relative change in gene expression before and after eight weeks of fenofibrate treatment

Table III-1. Plasma and lipoproteins parameters, expressed as mean ± S.E.M., at baseline (W0) and 4 weeks (W4) thereafter

	W0	W4	p value	n value
Triglycerides (mg/dL)	144.7 + 20.8	98.7 + 13.6	p < 0.00005	29
Cholesterol (mg/dL)	182.0 ± 6.0	158.7 ± 6.4	p <0.00005	29
HDL-C (mg/dL)	43.7 ± 2.1	45.0 ± 2.0	p <0.62	29
LDL-C (mg/dL)	110.5 ± 5.4	94.0 ±5.1	p < 0.00002	29
hsCRP (mg/L)	2.8 ± 0.4	1.8± 0.2	p < 0.70	23
sVCAM-1 (ng/mL)	704.6 ± 51.7	667.7 ± 34.2	p < 0.15	29

Table III-2. Comparison of individuals with elevated hsCRP and sVCAM-1 and individuals with elevated hsCRP or sVCAM-1, values expressed as mean ± S.E. M., at baseline week 0 (W0) and 4 weeks (W4) thereafter

	Individuals with elevated hsCRP and sVCAM-1			Individuals with elevated hsCRP or sVCAM-1		
	W0	**W4**	**p value**	**W0**	**W4**	**p value**
Bcl-6 gene expression (n-fold)	1*	0.864 ± 0.12	p < 0.03	1*	0.959 ± 0.14	p < 0.73
	n =17			n=12		
Spen gene expression (n-fold)	1*	0.928 ± 0.04	p <0.14	1*	0.727 ± 0.05	p < 0.004
	n =17			n=12		
hsCRP (mg/L)	3.4 ± 0.7	2.0 ± 0.4	p < 0.30	2.1 ± 0.5	1.5 ± 0.3	p < 0.20
	n=12			n=11		
sVCAM-1 (ng/mL)	794.7 ± 79.0	734.3 ± 51.5	p < 0.15	576.9 ± 31.6	573.8 ± 24.7	p < 0.90
	n=17			n=12		

* Pretreatment gene expression is normalized to 1*

Table III-3. Plasma and lipoproteins parameters, expressed as mean ± S.E.M., at baseline (W0), 4 weeks (W4), 6 weeks (W6), and 8 weeks (W8) thereafter

	W0	W4	W6	W8	p value, respectively	n value
Triglycerides (mg/dL)	167.8 ± 56.9	122.9 ± 32.4	103.8 ± 21.0	108.1 ± 24.2	$p < 0.09$, $p < 0.01$, $p < 0.003$	9
Cholesterol (mg/dL)	207.9 ± 5.7	190.1 ± 10.5	190.5 ± 7.3	188.5 ± 7.8	$p<0.02$,$p <0.003$, $p <0.02$	9
HDL-C (mg/dL)	45.5 ± 4.9	47.0 ± 4.2	49.8 ± 4.5	50.4 ± 5.1	$p < 0.42$, $p < 0.02$, $p < 0.05$	9
LDL-C (mg/dL)	135.9 ± 7.0	118.7 ± 7.9	120.5 ± 7.7	119.1 ± 6.8	$p < 0.003$, $p < 0.007$, $p < 0.05$	9
hsCRP (mg/L)	2.9 ± 0.8	2.3 ± 0.5	2.4 ± 0.6	2.9 ± 0.9	$p < 0.81$, $p <0.21$, $p < 0.93$	7
sVCAM-1 (ng/mL)	763.9 ± 154.4	734.4 ± 93.2	789.1 ± 116.2	785.5 ± 111.6	$p < 1$, $p < 0.65$, $p < 0.35$	9

Table III.4 Gene expression (n-fold), expressed as mean ± S.E.M., at baseline week 0 (W0), and 4 weeks (W4) thereafter, n =29

Gene	W0	W4	p value
Bcl-6	1*	0.903 ± 0.09	$p < 0.08$
Spen	1*	0.845 ± 0.05	$p < 0.003$

* Pretreatment gene expression is normalized to 1*

Table III.5 Gene expression (n-fold), expressed as mean ± S.E.M., at baseline week 0 (W0), 4 weeks (W4), 6 weeks (W6), and 8 weeks (W8) thereafter, n =9

Gene	W0	W4	W6	W8	p value, respectively
Bcl-6	1*	0.817 ± 0.13	0.757 ± 0.09	0.921 ± 0.11	$p < 0.12$, $p < 0.07$, $p < 0.25$
Spen	1*	0.866 ± 0.05	1.047 ± 0.13	0.964 ± 0.06	$p < 0.07$, $p < 0.73$, $p < 0.57$

* Pretreatment gene expression is normalized to 1*

IV DISCUSSION

Fenofibrate's molecular mechanism of lipid modulation is well understood. However, the mechanics of how this drug lowers inflammation has yet to be elucidated. The purpose of the study is to confirm the increased gene expression of Bcl-6 and Spen in response to fenofibrate treatment, both of which are hypothesized to play a role in the anti-inflammatory effects of fenofibrate. Thirty four subjects with elevated inflammatory markers (hsCRP, sVCAM-1) were recruited and took fenofibrate for either 4 or 8 weeks. Gene expression profiles were measured by real time PCR before and after treatment with fenofibrate. The study found that Bcl-6 and Spen gene expression is not increased by the study drug.

IV.1 Inflammatory markers

Our study investigated the expression of hsCRP in regards to treatment with fenofibrate. There was no change in hsCRP expression after treatment with fenofibrate. Subjects taking the drug for 8 weeks also did not show a significant decrease in any of the post treatment periods (4 weeks, 6 weeks, or 8 weeks). However, when subjects were separated into two groups based on their baseline hsCRP values, individuals in the second tile showed a significant decrease after treatment with fenofibrate ($p < 0.01$). Fenofibrate treatment studies have been inconsistent in regard to the efficacy in which they lower CRP values. Several studies have shown a significant decrease in hsCRP.[53-55] Conversely, several more studies, including GOLDN, have not produced a significant change in hsCRP.[56, 57] Further studies clearly need to be done to determine the role of hsCRP and the anti-inflammatory effects of fenofibrate.

In addition to hsCRP, our study also investigated the expression of sVCAM-1 in regards to treatment with fenofibrate. We did not find a significant decrease in sVCAM-1 after four weeks of treatment. Figure III-5A, sVCAM-1 data in quartiles, provides more insight into the change of expression in the soluble adhesion molecule. While the first two quartiles are not significant, the changes between pre-treatment and post-treatment samples in quartile three and four are significant ($p < 0.04$, $p < 0.01$). The first two quartiles show a slight decrease or slight increase in expression but quartile three and four shows a clear decrease in expression. These results indicate that the higher the baseline levels of VCAM the greater the decrease after treatment. This was also the case for hsCRP, where individuals with a higher baseline of hsCRP saw a significant decrease after treatment.

In our previous study GOLDN, a significant decrease of VCAM was seen in participants during a 3 week trial of fenofibrate. Both the current study and GOLDN demonstrated that the higher the baseline levels of VCAM, the greater the decrease after treatment. Other studies have also shown a decrease in VCAM as a consequence of fenofibrate medication. Marchesi et al. and Rosenson et al. both did three month trials of fenofibrate in hypertriglyceridemia populations and VCAM-1 was significantly reduced in both studies.[58, 59] Another three month trial done by Ryan et. al. in obese glucose tolerant men also showed a significant decrease in the soluble adhesion molecule.[60]

A small subset of patients was selected to participate in the extended study. These patients took the study drug for a total of 8 weeks. The purpose of the 8 week study was to test whether the inflammatory markers and gene expression were altered after additional drug and to provide insight into the time course of fenofibrate in regards

to inflammation and lipid modulation. After 8 weeks of medication, a further decrease of VCAM was expected. It was observed that VCAM does not continuously decrease in subjects who take the drug for 8 weeks, nor is the change significant. The pattern of change in VCAM is variable from subject to subject. The small population size in the current study could account for the lack of significant decrease in sVCAM-1 expression.

IV.2 Lipid profile

In addition to measuring inflammatory markers, lipid profiles were measured for each subject before and after treatment. After 4 weeks of treatment with fenofibrate there was a significant decrease in cholesterol, triglycerides, LDL-C. There was not a significant increase in HDL-C. However, during the 8 week subjects did show a significant decrease in HDL-C ($p<0.02$, $p<0.05$ at 6 weeks and 8 weeks, respectively), as well as a continuing significant decrease in cholesterol, triglycerides, and LDL-C. Historically, the purpose of fenofibrate is to reduce triglycerides and increase HDL, and the results from the present study have been confirmed by numerous other studies.[61-65]

IV.3 Gene expression

The purpose of this study was to confirm the increase of Bcl-6 and Spen expression after treatment with fenofibrate. Preliminary data from microarray gene expression analysis indicated a 1.8 fold increase in Bcl-6 expression and a 1.7 fold increase in Spen expression in 9 patient pairs after treatment with fenofibrate. Bcl-6 transcriptionally represses inflammatory molecules, including sVCAM-1, through interaction with PPAR-δ. After treatment with fenofibrate in the current study an increase in gene expression was expected in both Bcl-6 and Spen. However, a decrease was only found in Spen (0.845 ± 0.05,) after 4 weeks of treatment. Nine subjects

continued taking fenofibrate for an additional 4 weeks. Relative expression of Bcl-6 after 4, 6, 8 weeks is 0.817 ± 0.13, 0.757 ± 0.09, 0.921 ± 0.11, respectively; but none of these changes were significant. Spen relative expression was 0.866 ± 0.05, 1.047 ± 0.13, 0.964 ± 0.06 for 28, 42, and 56 days respectively. There were no significant changes for Spen found in these nine subjects. The increase of gene expression from the preliminary microarray data could not be confirmed by the present study.

Subjects in the current study fall into two groups: individuals with elevated hsCRP and sVCAM-1 or individuals with one elevated marker of inflammation. The disparity in our population is due to the change in inflammation markers between the screening and experimental phase. The time period between an individual's screening phase and experimental was highly variable. Seventeen of the 29 subjects had elevated hsCRP and sVCAM-1 at the start of taking the study drug. However, 12 subjects had only one elevated marker of inflammation at the beginning of experimental. These two groups are compared to establish similarities and differences.

The two groups are analogous in regards to hsCRP and sVCAM-1 expression in that both groups showed no change in either inflammatory marker after 28 days of treatment. The two groups differ in regards to gene expression. Individuals with elevated hsCRP and sVCAM-1 had a significant decrease in Bcl-6 and a non-significant decrease in Spen. Conversely, the subjects who only have one elevated marker of inflammation pre-treatment have a significant decrease in Spen and no change in Bcl-6. It is interesting that individuals who began the experimental phase with elevated hsCRP and sVCAM-1, have a significant decrease in Bcl-6 (0.864 ± 0.12) as 70% of the individuals in this group saw a decrease in sVCAM-1, despite the decrease in gene

expression. Rather, an increase in Bcl-6 expression to correlate with decreased sVCAM-1 would be expected. Fenofibrate does not appear to affect Spen in these individuals as there was no change in Spen gene expression. The opposite scenario is present for subjects who only had one elevated marker of inflammation at the beginning of the experimental phase. Bcl-6 and Spen were expressed at 0.959 ± 0.14 and 0.727 ± 0.05, respectively. There was no real change in Bcl-6 expression in these individuals, compared to a significant decrease in Spen.

We did not see an increase in Bcl-6 expression, nor was a significant decrease seen in sVCAM-1 in the 29 subjects in the present study. A comparison was done between subjects who showed the greatest increase and decrease in Bcl-6 expression in the 29 participants. Contradictory results were found in regards to sVCAM-1 expression. The inflammatory markers and lipid profile measurements were examined for the 6 subjects who showed the greatest increase in Bcl-6. Two thirds of these individuals showed an increase in sVCAM-1 after treatment with fenofibrate, where as one third showed an increase in CRP. General inflammation via CRP appears to be decreased in these individuals, however sVCAM-1 is increasing. Bcl-6 transcriptionally represses sVCAM-1 expression, so a decrease in sVCAM-1 was expected in individuals with an increase in Bcl-6 expression. Rather, an unexpected opposite effect is observed. These results are compounded by the results seen in individuals with the greatest decrease in Bcl-6 expression post treatment. All of the 6 subjects who showed the greatest decrease in Bcl-6 expression had a decrease in sVCAM-1 expression after treatment. Two thirds showed a decrease in hsCRP. We did not expect individuals with a decrease in Bcl-6 expression to show decrease in sVCAM-1. The lipid profile of these individuals did not

show anything unusual. These data indicate that Bcl-6 may not be involved in the anti-inflammatory effects of fenofibrate. The majority of these individuals are seeing a decrease in their CRP implying that general inflammation is being lowered by treatment with fenofibrate. If Bcl-6 was involved in the lowering of inflammation, the results should have been reversed between the two groups.

A large part of the hypothesis that Bcl-6 is involved in the anti-inflammatory effects of fenofibrate hinged on the idea that PPAR-δ would be activated by fenofibrate. It is well known that fenofibrate interacts with PPAR-α, however, thus far, studies have not shown a relationship between PPAR-δ and the study drug. It is likely that PPAR-δ is not a ligand for fenofibrate. The preliminary experiments that led to the current study could have also had underlying issues due to the limitations of microarrays. The preliminary experiment sample size and the current studies population are small, and small populations may not show differences due to their limited statistical power. This study also brings up the question of what is the normal expression of these two genes and what is the ideal cell type to study the effects of these genes. Individually or combinations of these issues could help to explain why we did not see the results we expected from the current study.

Microarray technology allows the simultaneous analysis of thousands of genes.[66] It presents the opportunity to investigate gene regulation and the changes in gene expression due to drug treatment or disease state. The design and process of microarrays is simple, however underneath the surface are many issues. Microarrays are a new technology and there is not a set methodology with standards and proper protocols. The greatest issue facing microarrays is that statistical analysis of microarray data is the exact

opposite of the data sets of which statisticians are accustomed. [67] Normally, data has many samples and a few numbers of variables; conversely, microarray data has a very large number of variables and a small number of samples. The results of experiments are generally reported only as fold change. This is problematic especially for low abundance transcripts. Affimetrix Genechips can vary by as much as 1000 raw fluorescence units in two identical samples. If a gene that is only expressed at 200 units, a 1000 unit variation will produce a 6 fold change.[68] The use of fold change is not ideal for genes that are expressed at low levels. Bcl-6 and Spen were found to be expressed at low levels, and the statistical analysis issues facing microarray technology reinforces the need to confirm microarray analysis results with other techniques.

In the previous study, microarray analysis was done on 19 subjects from the GOLDN study. After quality control, only 9 subjects were used for gene expression analysis. Four of those subjects clustered separately from before and after fenofibrate. Bcl-6 and Spen remained the genes that showed the most changes with regard to frequency and magnitude (3.4 and 2.7 fold, respectively). It seems likely that these four subjects could have inflated the fold change of the nine subject pairs, 1.8 and 1.7. The other five subjects did not cluster separately, indicating less of a change in gene expression before and after treatment. A larger population size would have a given a fold change that is less susceptible to a few individuals with a significant change in expression.

The current study also had a small population, 29 participants, 9 of which participated in the extended study. As the purpose of the current investigation was to elucidate the anti-inflammatory properties of fenofibrate, recruiting individuals with

elevated inflammatory markers was important. However, it also greatly limited the number of subjects in this study. The pool of patients to screen (n=284) did not have a large number of patients with elevated inflammatory markers. The majority of patients who did show elevated markers of inflammation were only mildly inflamed. Ideally, we would have recruited individuals with sVCAM-1 expression >800 ng/mL along with elevated CRP. Perhaps part of the reason that a significant decrease in sVCAM-1 was not seen in these subjects is due to the fact that they show only mild signs of inflammation. Of the subjects whose inflammatory markers remained elevated at the beginning of the treatment phase, only one subject had a sVCAM-1 value greater than 900 ng/mL. A larger population with individuals who have a greater elevation of inflammatory markers could potentially show the decrease in inflammation that was expected in the current study.

Microarray technology has given researchers the ability to measure the gene transcription profiles of thousands of genes simultaneously from different cells and tissue types. However, with the advent of this technology very few studies have been done to determine the pattern of gene expression of healthy individuals and the potential intra and inter-variation among these healthy subjects.[69] Normality is an important issue to address as "normal" gene expression will have variability. It will not be a set value but rather a range of values. There is currently not a normal range of expression known for Bcl-6 or Spen.

While a "normal" value range is not known for Bcl-6, studies have shown variation among healthy individuals. Bcl-6 was among 3,302 genes that varied significantly in transcript levels in healthy subjects in a microarray study using PBMCs

(peripheral blood mononuclear cells) done by Eady et al.[70] It was found that the expression of Bcl-6 correlated significantly with the percentage of monocytes present. A similar study done by Whitney et al found that Bcl-6 showed a strong correlation with neutrophils in the whole blood samples. PBMC samples were also taken in Whitney' study, and there was a significant difference of expression of Bcl-6 between PBMC and whole blood.[71] In addition, a study was done to determine normal variance in gene expression of mice. Genetically identical healthy mice were sacrificed and their organ tissues were used for microarray analysis. There was great variance in Bcl-6 gene expression in both the liver and kidney.[72]

Bcl-6 is ubiquitously expressed and in four different tissue types (kidney, liver, whole blood, and PBMCs) [69, 71, 72], it has shown variance among healthy individuals. More microarray studies need to be done involving healthy subjects, but thus far Bcl-6 appears to have variable gene expression in these individuals. While there was slight decrease in expression in Bcl-6, it has yet to be determined what the impact of that decrease has on an individual, given the lack of "normal" range of gene expression for Bcl-6. There have been no studies done to measure the normal range of Spen.

Peripheral blood leukocytes were used in the previous and current study to determine gene expression of Bcl-6 and Spen. However, leukocytes may not be the ideal cell type to study Bcl-6 gene expression. The study that demonstrated that Bcl-6 represses VCAM-1 via PPAR delta was done in vascular endothelial cells.[44] VCAM-1 is highly expressed in endothelial cells.[73, 74] In addition, fenofibrate has already been shown to decrease VCAM in the endothelium.[75] Acquiring human vascular endothelial cells, however, is an invasive procedure. It would be difficult to obtain informed consent from

research subjects to undergo such a procedure after treatment with fenofibrate. None the less, vascular endothelial cells could be a promising cell type to investigate the gene expression of Bcl-6 in regards to fenofibrate treatment.

Furthermore, Bcl-6 protein levels or protein activity were not measured in this study. While gene expression of Bcl-6 was measured, the level of gene expression is not always equivalent with the actual protein levels or activity. Protein level can be increased even if mRNA transcript is decreased or unchanged. Further investigation into the impact of fenofibrate treatment on the protein levels and subsequent activity of Bcl-6 is needed.

In summary, an increase in Bcl-6 and Spen expression could not be confirmed by the current study. This could be due to several limitations including the preliminary studies methods, the lack of understanding of what normal expression of these two genes are, and/or leukocytes may not be the ideal cell type to study Bcl-6 gene expression. Ultimately, the greatest limitation in this study was the small population size and the subsequent mild inflammation present in the population. Fenofibrate did decrease cholesterol, triglycerides, and LDL-C as well as increasing HDL-C in the subjects of this study, verifying the efficacy of treatment. This study also demonstrated there is a correlation between the baseline levels of inflammatory markers and the amount of decrease that is seen with fenofibrate treatment. A larger population with highly elevated inflammatory markers may show a different outcome than in the current investigation.

V. REFERENCES

1. Lloyd-Jones D et. al. American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics--2009 update: A report from the american heart association statistics committee and stroke statistics subcommittee. Circulation 2009 Jan 27;119(3):480-6.

2. Lloyd-Jones D et. al. American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics--2010 update: A report from the american heart association. Circulation 2010 Feb 23;121(7):e46-e215.

3. Libby P. Braunwald's heart disease : A textbook of cardiovascular medicine. 8th ed. Philadelphia, PA: Saunders Elsevier; 2008.

4. Zandberggen F, Plutzky J. PPARα in atherosclerosis and inflammation. Biochim Biophys Acta 2007;1771(8):972-82.

5. Pepys MB, Hirschfield GM. C-reactive protein: A critical update. J Clin Invest 2003 Jun;111(12):1805-12.

6. McGoon MD. Mayo clinic heart book. New York: W. Morrow; 1993.

7. Libby P. Atherosclerosis: The new view. Sci Am 2002 May;286(5):46-55.

8. Insull W,Jr. The pathology of atherosclerosis: Plaque development and plaque responses to medical treatment. Am J Med 2009 Jan;122(1 Suppl):S3-S14.

9. VCAM1 vascular cell adhesion molecule 1 [Homo sapiens] [Internet] [cited 2009 June/20]. Available from: http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&term=7412.

10. Nakashima Y, Raines EW, Plump AS, Breslow JL, Ross R. Upregulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the ApoE-deficient mouse. Arterioscler Thromb Vasc Biol 1998 May;18(5):842-51.

11. Cybulsky MI, Iiyama K, Li H, Zhu S, Chen M, Iiyama M, Davis V, Gutierrez-Ramos JC, Connelly PW, Milstone DS. A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. J Clin Invest 2001 May;107(10):1255-62.

12. Dennis EA. Diversity of group types, regulation, and function of phospholipase A2. J Biol Chem 1994 May 6;269(18):13057-60.

13. Mohler ER,3rd, Ballantyne CM, Davidson MH, Hanefeld M, Ruilope LM, Johnson JL, Zalewski A, Darapladib Investigators. The effect of darapladib on plasma

lipoprotein-associated phospholipase A2 activity and cardiovascular biomarkers in patients with stable coronary heart disease or coronary heart disease risk equivalent: The results of a multicenter, randomized, double-blind, placebo-controlled study. J Am Coll Cardiol 2008 Apr 29;51(17):1632-41.

14. Zalewski A, Macphee C. Role of lipoprotein-associated phospholipase A2 in atherosclerosis: Biology, epidemiology, and possible therapeutic target. Arterioscler Thromb Vasc Biol 2005 May;25(5):923-31.

15. Harizi H, Corcuff JB, Gualde N. Arachidonic-acid-derived eicosanoids: Roles in biology and immunopathology. Trends Mol Med 2008 Oct;14(10):461-9.

16. Gilmore TD. Introduction to NF-kappaB: Players, pathways, perspectives. Oncogene 2006 Oct 30;25(51):6680-4.

17. Hayden MS, West AP, Ghosh S. NF-kappaB and the immune response. Oncogene 2006 Oct 30;25(51):6758-80.

18. Brasier AR. The NF-kappaB regulatory network. Cardiovasc Toxicol 2006;6(2):111-30.

19. Ridker PM. Clinical application of C-reactive protein for cardiovascular disease detection and prevention. Circulation 2003 Jan 28;107(3):363-9.

20. Wilson PW, Pencina M, Jacques P, Selhub J, D'Agostino R Sr, O'Donnell CJ. C-reactive protein and reclassification of cardiovascular risk in the framingham heart study. Circ Cardiovasc Qual Outcomes 2008;1(2):92-7.

21. Wilson AM, Ryan MC, Boyle AJ. The novel role of C-reactive protein in cardiovascular disease: Risk marker or pathogen. Int J Cardiol 2006 Jan 26;106(3):291-7.

22. Asher J, Houston M. Statins and C-reactive protein levels. J Clin Hypertens (Greenwich) 2007 Aug;9(8):622-8.

23. Angles-Cano E. How statins and fibrates lower CRP. Blood;103(11):3996-7.

24. Kleemann R, Verschuren L, de Rooij B, Lindeman J, de Maat MM, Szalai AJ, Princen HMG, Koositra T. Evidence for anti-iinflammatory activity of statins and PPAR alpha activators in human C-reactive protein transgenic mice in vivo and in cultured human hepatocytes in vitro. Blood 2004;103(11):4188-94.

25. Keating GM, Croom KF. Fenofibrate: A review of its use in primary dyslipidaemia, the metabolic syndrome and type 2 diabetes mellitus. Drugs 2007;67(1):121-53.

26. Gaw,A.,Packard,C.J.,Sheperd,J. Fibrates. In: Principles and treatment of lipoprotein disorders. Berlin: Springer-Verlag; 1994.

27. Tsimihodimos V, Miltiadous G, Daskalopoulou SS, Mikhailidis DP, Elisaf MS. Fenofibrate: Metabolic and pleiotropic effects. Curr Vasc Pharmacol 2005 Jan;3(1):87-98.

28. Schoonjans K, Staels B, Auwerx J. Role of the peroxisome proliferator-activated receptor (PPAR) in mediating the effects of fibrates and fatty acids on gene expression. J Lipid Res 1996 May;37(5):907-25.

29. Tsimihodimos V, Liberopoulos E, Elisaf M. Pleiotropic effects of fenofibrate. Curr Pharm Des 2009;15(5):517-28.

30. Fruchart JC, Duriez P. Mode of action of fibrates in the regulation of triglyceride and HDL-cholesterol metabolism. Drugs Today (Barc) 2006 Jan;42(1):39-64.

31. Lee CH, Chawla A, Urbiztondo N, Liao D, Boisvert WA, Evans RM, Curtiss LK. Transcriptional repression of atherogenic inflammation: Modulation by PPARdelta. Science 2003 Oct 17;302(5644):453-7.

32. Duval C, Chinetti G, Trottein F, Fruchart JC, Staels B. The role of PPARs in atherosclerosis. Trends Mol Med 2002 Sep;8(9):422-30.

33. Michalik L, Auwerx J, Berger JP, Chatterjee VK, Glass CK, Gonzalez FJ, Grimaldi PA, Kadowaki T, Lazar MA, O'Rahilly S, Palmer CN, Plutzky J, Reddy JK, Spiegelman BM, Staels B, Wahli W. International union of pharmacology. LXI. peroxisome proliferator-activated receptors. Pharmacol Rev 2006 Dec;58(4):726-41.

34. Feige JN, Gelman L, Michalik L, Desvergne B, Wahli W. From molecular action to physiological outputs: Peroxisome proliferator-activated receptors are nuclear receptors at the crossroads of key cellular functions. Prog Lipid Res 2006 Mar;45(2):120-59.

35. Berger J, Moller DE. The mechanisms of action of PPARs. Annu Rev Med 2002;53:409-35.

36. Oliver WR,Jr, Shenk JL, Snaith MR, Russell CS, Plunket KD, Bodkin NL, Lewis MC, Winegar DA, Sznaidman ML, Lambert MH, Xu HE, Sternbach DD, Kliewer SA, Hansen BC, Willson TM. A selective peroxisome proliferator-activated receptor delta agonist promotes reverse cholesterol transport. Proc Natl Acad Sci U S A 2001 Apr 24;98(9):5306-11.

37. Shi Y, Hon M, Evans RM. The peroxisome proliferator-activated receptor delta, an integrator of transcriptional repression and nuclear receptor signaling. Proc Natl Acad Sci U S A 2002 Mar 5;99(5):2613-8.

38. Cao J, Tsai MY, Zhang H, Nossova N, Arnett DK, Hanson NQ, Liew CC. Microarray analysis of peripheral lymphocytes before and after fenofibrate treatment in hypertrigylceridemic individuals-the GOLDN study. 48th Cardiovascular Disease Epidemiology and Prevention- and -Nutrition, Physical Activity, and Metabolism Conference 2008.;Lipids & Lipoproteins P12.

39. B-cell CLL/lymphoma 6 [Internet] [cited 2009 June/20]. Available from: http://www.genecards.org/cgi-bin/carddisp.pl?gene=BCL6.

40. Stogios PJ, Downs GS, Jauhal JJ, Nandra SK, Prive GG. Sequence and structural analysis of BTB domain proteins. Genome Biol 2005;6(10):R82.

41. Ci W, Polo JM, Melnick A. B-cell lymphoma 6 and the molecular pathogenesis of diffuse large B-cell lymphoma. Curr Opin Hematol 2008 Jul;15(4):381-90.

42. Harris MB, Mostecki J, Rothman PB. Repression of an interleukin-4-responsive promoter requires cooperative BCL-6 function. J Biol Chem 2005 Apr 1;280(13):13114-21.

43. BTB Domain Database [Internet]; cSeptember 13, 2007 [cited 2009 June 18]. Available from: http://btb.uhnres.utoronto.ca/.

44. Fan Y, Wang Y, Tang Z, Zhang H, Qin X, Zhu Y, Guan Y, Wang X, Staels B, Chien S, Wang N. Suppression of pro-inflammatory adhesion molecules by PPAR-delta in human vascular endothelial cells. Arterioscler Thromb Vasc Biol 2008 Feb;28(2):315-21.

45. Li Z, Wang X, Yu RY, Ding BB, Yu JJ, Dai XM, Naganuma A, Stanley ER, Ye BH. BCL-6 negatively regulates expression of the NF-kappaB1 p105/p50 subunit. J Immunol 2005 Jan 1;174(1):205-14.

46. Toney LM, Cattoretti G, Graf JA, Merghoub T, Pandolfi PP, Dalla-Favera R, Ye BH, Dent AL. BCL-6 regulates chemokine gene transcription in macrophages. Nat Immunol 2000 Sep;1(3):214-20.

47. Plutzky J. Medicine. PPARs as therapeutic targets: Reverse cardiology? Science 2003 Oct 17;302(5644):406-7.

48. Ravaux L, Denoyelle C, Monne C, Limon I, Raymondjean M, El Hadri K. Inhibition of interleukin-1beta-induced group IIA secretory phospholipase A2 expression by peroxisome proliferator-activated receptors (PPARs) in rat vascular smooth muscle cells: Cooperation between PPARbeta and the proto-oncogene BCL-6. Mol Cell Biol 2007 Dec;27(23):8374-87.

49. Ariyoshi M, Schwabe JW. A conserved structural motif reveals the essential transcriptional repression function of spen proteins and their role in developmental signaling. Genes Dev 2003 Aug 1;17(15):1909-20.

50. Shi Y, Downes M, Xie W, Kao HY, Ordentlich P, Tsai CC, Hon M, Evans RM. Sharp, an inducible cofactor that integrates nuclear receptor repression and activation. Genes Dev 2001 May 1;15(9):1140-51.

51. Arici M, Walls J. End-stage renal disease, atherosclerosis, and cardiovascular mortality: Is C-reactive protein the missing link? Kidney Int 2001 Feb;59(2):407-14.

52. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001 May 1;29(9):e45.

53. Coban E, Ozdogan M, Yazicioglu G, Sari R. The effect of fenofibrate on the levels of high sensitivity C-reactive protein in dyslipidaemic hypertensive patients. Int J Clin Pract 2005 Apr;59(4):415-8.

54. Wu TJ, Ou HY, Chou CW, Hsiao SH, Lin CY, Kao PC. Decrease in inflammatory cardiovascular risk markers in hyperlipidemic diabetic patients treated with fenofibrate. Ann Clin Lab Sci 2007 Spring;37(2):158-66.

55. Ye P, Li JJ, Su G, Zhang C. Effects of fenofibrate on inflammatory cytokines and blood pressure in patients with hypertriglyceridemia. Clin Chim Acta 2005 Jun;356(1-2):229-32.

56. Kim CJ. Effects of fenofibrate on C-reactive protein levels in hypertriglyceridemic patients. J Cardiovasc Pharmacol 2006 Jun;47(6):758-63.

57. Kon Koh K, Yeal Ahn J, Hwan Han S, Kyu Jin D, Sik Kim H, Cheon Lee K, Kyun Shin E, Sakuma I. Effects of fenofibrate on lipoproteins, vasomotor function, and serological markers of inflammation, plaque stabilization, and hemostasis. Atherosclerosis 2004 Jun;174(2):379-83.

58. Marchesi S, Lupattelli G, Lombardini R, Roscini AR, Siepi D, Vaudo G, Pirro M, Sinzinger H, Schillaci G, Mannarino E. Effects of fenofibrate on endothelial function and cell adhesion molecules during post-prandial lipemia in hypertriglyceridemia. J Clin Pharm Ther 2003 Oct;28(5):419-24.

59. Rosenson RS, Wolff DA, Huskin AL, Helenowski IB, Rademaker AW. Fenofibrate therapy ameliorates fasting and postprandial lipoproteinemia, oxidative stress, and the inflammatory response in subjects with hypertriglyceridemia and the metabolic syndrome. Diabetes Care 2007 Aug;30(8):1945-51.

60. Ryan KE, McCance DR, Powell L, McMahon R, Trimble ER. Fenofibrate and pioglitazone improve endothelial function and reduce arterial stiffness in obese glucose tolerant men. Atherosclerosis 2007 Oct;194(2):e123-30.

61. Knopp RH. Review of the effects of fenofibrate on lipoproteins, apoproteins, and bile saturation: US studies. Cardiology 1989;76 Suppl 1:14,22; discussion 29-32.

62. Knopp RH, Brown WV, Dujovne CA, Farquhar JW, Feldman EB, Goldberg AC, Grundy SM, Lasser NL, Mellies MJ, Palmer RH. Effects of fenofibrate on plasma lipoproteins in hypercholesterolemia and combined hyperlipidemia. Am J Med 1987 Nov 27;83(5B):50-9.

63. Krempf M, Rohmer V, Farnier M, Issa-Sayegh M, Corda C, Sirugue I, Gerlinger C, Masseyeff-Elbaz MF. Efficacy and safety of micronised fenofibrate in a randomised double-blind study comparing four doses from 200 mg to 400 mg daily with placebo in patients with hypercholesterolemia. Diabetes Metab 2000 May;26(3):184-91.

64. Mellies MJ, Stein EA, Khoury P, Lamkin G, Glueck CJ. Effects of fenofibrate on lipids, lipoproteins, and apolipoproteins in 33 subjects with primary hypercholesterolemia. Atherosclerosis 1987 Jan;63(1):57-64.

65. O'Connor P, Feely J, Shepherd J. Lipid lowering drugs. BMJ 1990 Mar 10;300(6725):667-72.

66. Hofman P. DNA microarrays. Nephron Physiol 2005;99(3):p85-9.

67. Murphy D. Gene expression studies using microarrays: Principles, problems, and prospects. Adv Physiol Educ 2002 Dec;26(1-4):256-70.

68. Ness SA. Microarray analysis: Basic strategies for successful experiments. Mol Biotechnol 2007 Jul;36(3):205-19.

69. Radich JP, Mao M, Stepaniants S, Biery M, Castle J, Ward T, Schimmack G, Kobayashi S, Carleton M, Lampe J, Linsley PS. Individual-specific variation of gene expression in peripheral blood leukocytes. Genomics 2004 Jun;83(6):980-8.

70. Eady JJ, Wortley GM, Wormstone YM, Hughes JC, Astley SB, Foxall RJ, Doleman JF, Elliott RM. Variation in gene expression profiles of peripheral blood mononuclear cells from healthy volunteers. Physiol Genomics 2005 Aug 11;22(3):402-11.

71. Whitney AR, Diehn M, Popper SJ, Alizadeh AA, Boldrick JC, Relman DA, Brown PO. Individuality and variation in gene expression patterns in human blood. Proc Natl Acad Sci U S A 2003 Feb 18;100(4):1896-901.

72. Pritchard CC, Hsu L, Delrow J, Nelson PS. Project normal: Defining normal variance in mouse gene expression. Proc Natl Acad Sci U S A 2001 Nov 6;98(23):13266-71.

73. Calabresi L, Gomaraschi M, Villa B, Omoboni L, Dmitrieff C, Franceschini G. Elevated soluble cellular adhesion molecules in subjects with low HDL-cholesterol. Arterioscler Thromb Vasc Biol 2002 Apr 1;22(4):656-61.

74. Ley K, Huo Y. VCAM-1 is critical in atherosclerosis. J Clin Invest 2001;107(10):1209.

75. Jun. W., Jinchao L, Zhaochu H, Biru O, Haisen G. Effect of hyperlipidemia on endothelial VCAM-1 expression and the protective role of fenofibrate. Frontiers of Medicine in China 2007;1(4):356-8.