

The Effects of Dietary Flaxseed on Atherosclerotic Plaque Regression

by

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Abstract

Dietary flaxseed intake has exhibited both cardioprotective and anti-atherogenic properties. Regardless, it remains unclear whether these beneficial effects extend to the regression of atherosclerotic plaques or the resolution of cholesterol-induced vascular contractile dysfunction. In the present study, we intended to determine whether dietary flaxseed has the capacity to ameliorate vascular function abnormalities and induce atherosclerotic plaque regression. As results from previous studies using a nutritional intervention to induce atherosclerotic regression may have been confounded by premature initiation of the intervention, an appropriate feeding regimen was developed to adequately evaluate flaxseeds' effects on atherosclerotic plaque regression. New Zealand white rabbits were utilized in two studies. To establish clear evidence of plaque growth stabilization, animals received 4 weeks of a 1% cholesterol-supplemented diet. An initial subset of animals was immediately examined. The remaining animals were fed regular rabbit chow and examined at intervals up to 28 weeks. To ascertain flaxseeds' effects on atherosclerotic plaque regression and vascular contractile function, animals were randomly assigned to a control group fed a regular diet for 12 weeks (Group I) or an experimental group fed a 1% cholesterol-supplemented diet for 4 weeks followed by a regular diet for 8 weeks (Group II). The control and a subset of experimental animals were examined immediately afterwards. The remaining experimental animals were given an additional 8 or 14 weeks of either a regular diet (Group III and V, respectively) or a 10% flaxseed-supplemented diet (Group IV and VI, respectively) and were examined afterwards. Cholesterol feeding followed by 8 weeks of withdrawal from cholesterol not only resulted in the development and stabilization of atherosclerotic plaques but also

impaired the maximum contraction caused by norepinephrine and the relaxation response to acetylcholine. An additional 14 weeks of regular diet reduced the amount of plaques on the aorta while flax-supplementation resulted in a further reduction in plaques. Nevertheless, both treatments were unable to achieve statistical significance. Flax-supplemented and regular diets improved vessel relaxation and contraction; however, negligible changes in the relaxation response induced by sodium nitroprusside were observed. Dietary flaxseed may accelerate the regression of atherosclerotic plaques. Moreover, the known beneficial effects of flaxseed do not extend to restoration of vascular function.

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To my loving father (Azariah Francis),
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“I sustain myself with the love of family.”

- Maya Angelou

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Abbreviations

AA = arachidonic acid

ABC = ATP-binding cassette transporter

Ach = acetylcholine

AHA = American Health Association

ALA = α -linolenic acid

Apo = apolipoprotein

ATP = adenosine triphosphate

CAD = coronary artery disease

cAMP = cyclic adenosine 3',5' -monophosphate

CCR7 = chemokine (C-C motif) receptor 7

cGMP = cyclic guanosine 3',5'- monophosphate

COX = cyclooxygenase

CVD = cardiovascular disease

DAG = diacylglycerol

DHA = docosahexaenoic acid

DPA = docosapentaenoic acid

EC = endothelial cell

ED = enterodiol

ENL = enterolactone

EPA = eicosapentaenoic acid

EPC = endothelial progenitor cell

FC = free cholesterol

FLC = flax lignan complex
GC = gas chromatography
G-CSF = granulocyte colony-stimulating factor
GPCR = G-protein coupled receptor
GTP = guanosine-5'-triphosphate
HDL = high-density lipoprotein
HRP = horseradish peroxidase
ICAM = intercellular adhesion molecules
IL = interleukin
IMT = intimal medial thickness
IP3 = inositol triphosphate
LA = linoleic acid
LDL = low-density lipoprotein
LOX = lipoxygenase
LT = leukotriene
MLC = myosin light chain
MLCK = myosin light chain kinase
MMP = matrix metalloproteinase
MUFA = monounsaturated fatty acid
NE = norepinephrine
NF- κ B = nuclear factor κ B
NO = nitric oxide
NCX = Na⁺/Ca²⁺ exchanger

NZW = New Zealand white

OCT = optical cutting temperature

PBS = phosphate buffered saline

PCNA = proliferating cell nuclear antigen

PFA = paraformaldehyde

PG = prostaglandin

PLC = phospholipase C

PMCA = plasma membrane Ca^{2+} -ATPase

PPAR = peroxisome proliferator-activated receptor

PUFA = polyunsaturated fatty acid

RCT = reverse cholesterol transport

SDG = secoisolariciresinol diglucoside

SECO = secoisolariciresinol

SERCA = sarco/endoplasmic reticulum Ca^{2+} -ATPase

SL = sarcolemma

SMC = smooth muscle cells

SNP = sodium nitroprusside

SDF-1 = stromal derived factor 1

SR = sarcoplasmic reticulum

SR-BI = scavenger receptor class B type I

TNF- α = tumor necrosis factor - α

TX = thromboxane

VLDL = very low-density- lipoprotein

VSMC = vascular smooth muscle cell

VWV = vessel wall volume

Introduction

Atherosclerosis is a chronic, progressive cardiovascular disease (CVD) that emerged as a major health dilemma during the early twentieth century. Nearly a full century later, it has become the leading cause of morbidity and mortality worldwide [1, 2]. Despite improved treatments for atherosclerosis, its prevalence continues to increase due to the escalating predominance of sedentary, unhealthy lifestyles [3-5].

Atherosclerosis is a diffuse disease that is orchestrated by a complex array of molecular and cellular events commencing after endothelial injury. The resultant plaques initially progress asymptotically, but as plaque development exacerbates they may manifest clinically with acute events such as myocardial infarction and stroke [3, 6]. Atherosclerotic plaques are known to be highly dynamic and their progression can be slowed, stopped and even reversed by drug intervention [7, 8]. For example, aggressive risk modification with statin drug therapy can induce the three processes involved in atherosclerotic plaque regression [9, 10].

Akin to pharmaceuticals, functional foods can also be used to prevent and treat CVD. Functional foods, such as flaxseed, have increased in popularity and are now considered to be a viable therapeutic strategy for CVD. Flaxseed contains one of the highest contents of α -linolenic acid (ALA), an ω -3 polyunsaturated fatty acid (PUFA) linked with CVD prevention [11-13]. Compared to various drug therapies, ω -3 PUFAs have fewer and milder side effects and are generally considered safe [14]. Moreover, akin to various pharmaceuticals, milled flaxseed has proven to be cardioprotective and anti-atherogenic in animal models [6, 15, 16] as well as in clinical settings [17].

Although flaxseed can diminish the progression of atherosclerotic plaques, it is unclear whether its supplementation may regress established atherosclerotic plaques.

Although many studies have utilized nutritional interventions to induce plaque regression [18-22], these studies have all prematurely initiated their interventions before there is clear evidence of cessation of plaque growth. If a rabbit is supplemented with regular rabbit chow followed by the withdrawal of cholesterol from the diet, atherosclerotic lesions actively continue to progress for many weeks. These lesions only begin to stabilize after an extended period of cholesterol withdrawal from the diet, when hypercholesterolemia has nearly resolved [23-27]. Thus, if an intervention is imposed immediately after the withdrawal of cholesterol from the diet as has been done in previous studies of regression [18-21, 28], any effects observed will reflect an inhibition of plaque progression and not an effect on plaque regression.

Thus, the objectives of the present study were to 1) identify a suitable dietary regimen in rabbits which showed clear evidence of plaque growth stabilization, 2) determine the ability of dietary flaxseed to induce or accelerate atherosclerotic plaque regression, and 3) discern whether flaxseed supplementation can reverse any cholesterol-induced vascular contractile abnormalities.

Review of Literature

1. Arterial structure

The circulatory system is a network of vessels responsible for the transportation, distribution and exchange of substances within blood throughout the body. Following systole, oxygenated blood from the left ventricle first enters the aorta, the largest mammalian artery, and is subsequently conducted through the arterial vasculature, supplying all tissues and organs of the body [29]. Generally, arteries contain connective, elastic and muscle tissue arranged in discrete layers; however their histological proportions change as arteries approach the periphery, before the transition to arterioles. The narrowing of branches and thinning of the arterial walls is accompanied with decreases in elastic tissue and an increase of vascular smooth muscle cells (VSMC) [29].

All arteries contain three distinct histological layers (Figure 1): the tunica intima, the tunica media, and the tunica adventitia [30]. The innermost layer of an artery is the tunica intima. The tunica intima is composed of a basement membrane adjoined to a monolayer of endothelial cells that lines the arterial lumen. Thus, the tunica intima's endothelial layer is in direct contact with blood and is important for vessel function and homeostasis. The tunica intima and its adjacent layer, the tunica media, is separated by the internal elastic lamina, a sub-endothelial cavity fortified with extracellular matrix that allows the diffusion of metabolites and substances to and from the tunica media [30].

The middle layer of arteries, the tunica media, consists predominantly of VSMCs and has intuitively been entitled the muscular layer. Additionally, the tunica media contains a supportive matrix of elastin and collagen that serves as a scaffold that orients VSMCs. The tunica media and the outermost layer, the tunica adventitia, is separated

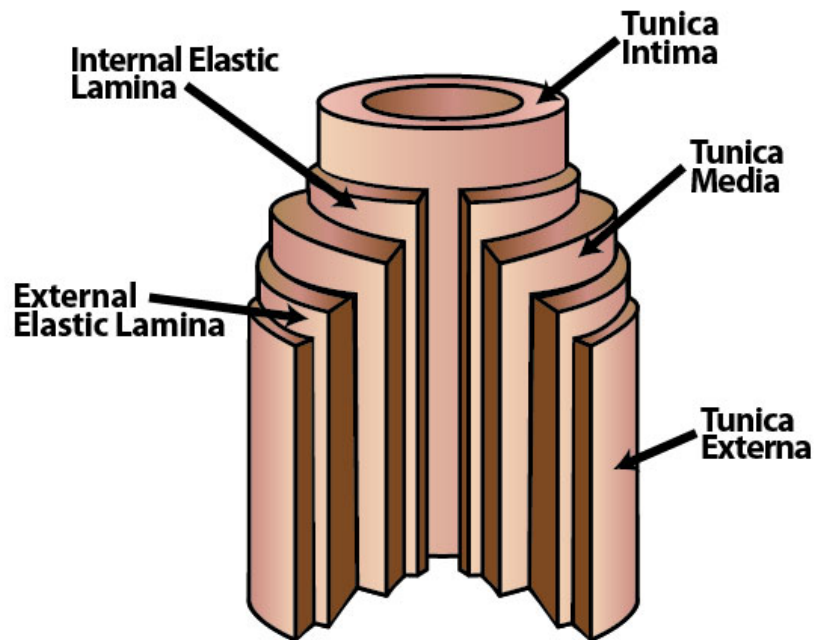


Figure 1. The histological structure of an artery.

by the external elastic lamina, which akin to the internal elastic lamina allows for the diffusion of substances between both layers [30].

The tunica adventitia is predominantly a conglomerate of fibroblasts, collagen and elastin that form the external covering of arteries. This layer also contains the *vaso vasorum*, a network of innervations and lymph and blood vessels that permeate and supply the tunica adventitia [30].

2. *Physiological function of arteries*

Endothelial cells (EC) and VSMCs are the primary functional components of arteries that are responsible for vessel function and homeostasis.

2.1 *Endothelial function*

Our understanding of the vascular endothelium has radically shifted from the 1960's view of it merely being an inert, semipermeable barrier that separates the interstitium from blood [31, 32]. This shift in perspective initially began when Furchgott and Zawadzki [33] used a rabbit thoracic aorta preparation to demonstrate how an intact endothelium can release an endothelium-derived relaxant factor that was responsible for acetylcholine (ACh)-dependent vasodilatation. Seven years later, Palmer and colleagues [34] discovered that this endothelium-derived relaxant factor was, in fact, nitric oxide (NO). Since these early pioneering studies, the endothelium is now recognized as a dynamic barrier that, under physiological conditions, control vascular homeostasis through the secretion of a myriad of substances that control vascular tone, blood pressure, leukocyte trafficking, and the antithrombotic and anticoagulant balance [32, 35].

2.2 *Vascular smooth muscle cell function*

Vascular smooth muscle cells are tonic smooth muscle cells that contract as a single unit after contraction is initiated pharmacologically or mechanically [29, 36]. Like all smooth muscle cells, VSMCs are mononuclear and their contractile apparatus, containing integrated thick and thin filaments connected to dense bodies, is arranged obliquely, as opposed to in uniform transverse alignments like skeletal and cardiac muscles. This arrangement provides smooth muscle cells with their characteristic non-striated "smooth" appearance.

VSMCs are electrically, mechanically and chemically coupled to one another, conferring the functionality of many cells acting as a single unit. Dense bodies within VSMCs are held in place by an intermediate filament scaffold comprised of vimentin and

desmin. In addition, adherens and gap junction fasten individual cells to one another. Thus, contractile force generation is permeated throughout the entire cell as well as through adjacent cells. In blood vessels, VSMCs are arranged circumferentially; therefore, contraction and relaxation will result in reductions and increases in vessel luminal diameter, respectively [29].

Various stimuli can initiate VSMC contractile activity through pharmacomechanical or mechanochemical (electromechanical) coupling. The former coupling pathway induces contraction (vasoconstriction) in response to an agent that may not produce changes in membrane potential. On the other hand, mechanochemical (electromechanical) coupling stimulates contraction through plasma membrane (sarcolemma) depolarization by mechanical or electrical means (Figure 2). Regardless, both pathways mediate contractile activity by elevating intercellular Ca^{2+} .

Amidst the proposed pharmacomechanical coupling mechanisms, the most common and well known involves activation of the phosphatidylinositol cascade [37]. The majority of VSMC contractile agonists, like norepinephrine (NE), serve as ligands for guanosine-5'-triphosphate (GTP)-binding protein (G-protein) coupled receptors (GPCR), such as the α 1-adrenergic receptor.

Stimulation of the α 1-adrenergic receptor by NE causes the α unit of a trimeric G-protein to exchange guanosine-5'-diphosphate for GTP. After the hydrolysis of GTP, the α -subunit activates phospholipase C (PLC), which subsequently catalyzes the splitting of sarcolemmal (SL) phospholipid phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). While DAG remains within the plasma membrane, IP3 diffuses through the cytosol and stimulates IP3 sensitive calcium chann-

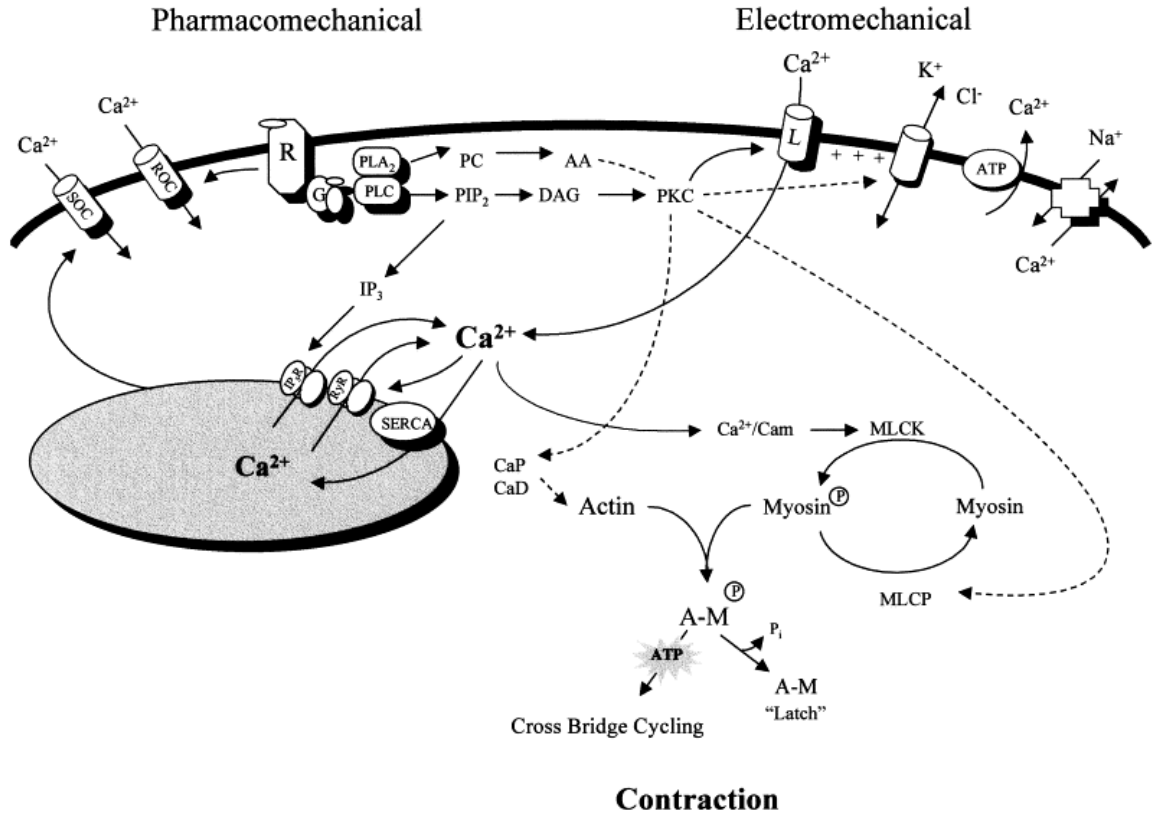


Figure 2. Cellular events involved in mechanochemical (electromechanical) and pharmacomechanical coupling. © 1999 MS Taylor, McMahon AM, JD Gardner, and JN Benoit. *Inflammation in Atherosclerosis: From Cyclic nucleotides and vasoconstrictor function: physiological and pathophysiological considerations. Pathophysiology.* Image used with permission from Elsevier on July 3, 2012 [38].

els on the sarcoplasmic reticulum (SR), mediating a release of Ca^{2+} [29, 37].

The influx of calcium ions from the extracellular fluid may also initiate contraction through mechanochemical coupling. Sarcolemmal Ca^{2+} channel activation is the major type of extracellular Ca^{2+} entry into the cytosol. In addition, several types of sarcolemmal Ca^{2+} channels exist in VSMCs, including two types of voltage-operated Ca^{2+} channels (“long-lasting” L-type and “transient” T-type Ca^{2+} channels), store-operated Ca^{2+} channels, and receptor-operated Ca^{2+} channels. The resulting increase in intracellular $[\text{Ca}^{2+}]$ from channel activation is not sufficient to induce VSMC contraction;

rather such “trigger” or “activatory” Ca^{2+} is used to activate Ca^{2+} -sensitive Ca^{2+} channels such as ryanodine receptors located on the SR, a process name calcium-induced calcium release [29, 37].

Irrespective of the mode of Ca^{2+} cytosolic elevation, this increase leads to the activation of motor proteins responsible for generating the force required for contraction. At high intracellular $[\text{Ca}^{2+}]$ four calcium ions complexes with calmodulin, inducing a conformational change that allows it to bind and activate myosin light chain kinase (MLCK). This calcium-calmodulin-MLCK complex phosphorylates serine 19 of the 20-kD regulatory myosin light chain (MLC), initiating muscle contraction according to the sliding filament theory (Figure 2) [29, 37].

Although the primary mechanism for VSMC contraction is $[\text{Ca}^{2+}]$ dependent, the relationship between the force of contraction from MLC phosphorylation and $[\text{Ca}^{2+}]$ can be adjusted, thus augmenting the “ Ca^{2+} sensitivity” of MLC phosphorylation [29, 39]. Several receptor hormones and agonists, including catecholamines, vasopressin, endothelin, angiotensin, and muscarinic agonists, can increase Ca^{2+} sensitization directly or indirectly through the RoA-kinase mediated inhibition of myosin phosphatase, which dephosphorylates serine 19 of the 20-kD regulatory MLC, or through CPI-17 phosphorylation by Rho-Kinase, respectively [29, 39].

Vascular smooth muscle cell relaxation (vasodilation) is equally important as contraction and is achieved through the reduction of cytosolic free $[\text{Ca}^{2+}]$. VSMCs have two Ca^{2+} ATPases (Ca^{2+} pump), one specific to the SL (PMCA) and another specific to the SR (SERCA), the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX), and cytosolic Ca^{2+} -binding proteins that all collaborate to decrease the cytosolic concentration of free Ca^{2+} . Regardless, only

the Ca^{2+} pumps are believed to have a pivotal role in cytosolic Ca^{2+} reduction. Moreover, only SERCA can “recharge” VSMC for contraction by sequestering Ca^{2+} within the SR [40].

A number of agents are known to activate these cytosolic efflux mechanisms in VSMCs. Most notable are the cyclic nucleotides, cyclic guanosine 3',5'- monophosphate (cGMP) or cyclic adenosine 3',5'- monophosphate (cAMP) (Figure 3). For example, Ach causes the activation of soluble NO synthase within ECs [29, 39]. Once produced NO immediately traverses the intimal space and internal elastic lamina to the VSMC of the tunica media, an event that is relatively localized given the half-life of NO and the proximity of VSMCs [41]. Nitric oxide induces the activation of membrane-bound guanylyl cyclase to increase cGMP in VSMCs, thereby activating cGMP-dependent protein kinase (protein kinase G). Protein kinase G has been implicated in activation of SERCA, PMCA, and NCX as well as the inhibition of both SL and SR Ca^{2+} channels and SL hyperpolarization [37, 39].

3. *Pathology of atherosclerosis*

Endothelial cells and VSMCs are essential for regulating normal homeostatic functions of the vasculature. During atherosclerosis, however, these cells become dysfunction and initiate as well as exacerbate atherogenesis. Moreover, additional cell types such as immune cells, which are essential to mammalian adaptive immunity, directly cause plaque formation and aid in orchestrating and perpetuating atherosclerotic plaque development and destabilization (Figure 4).

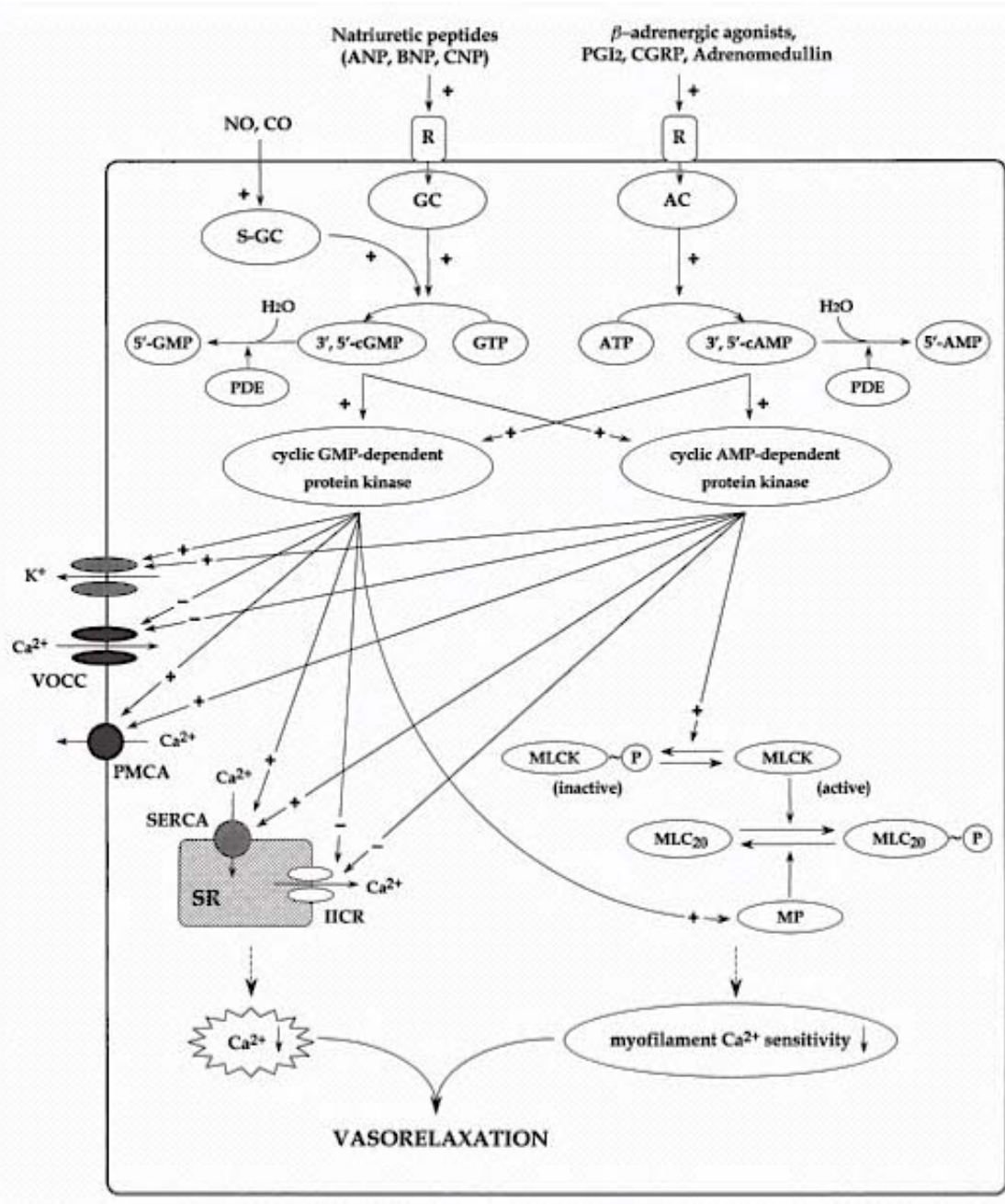


Figure 3. Mechanisms of vascular smooth muscle cell relaxation. © 2007 T Akata. Cellular and molecular mechanisms regulating vascular tone. Part 2: regulatory mechanisms modulating Ca²⁺ mobilization and/or myofilament Ca²⁺ sensitivity in vascular smooth muscle cells. *Journal of Anesthesia*. Image used with permission from Springer on July 3, 2012 [37]

3.1 Endothelial dysfunction

C. von Rokitansky and R. Virchow first proposed the endothelial response to injury theory of atherosclerosis during the mid-1800s [42]. Over a century later, in 1986, Dr. R. Ross' [43] improvement and validation of the response to injury theory has transformed this theory into a well accepted dogma [44]. It is now apparent that atherosclerosis is initiated by ECs response to various noxious stimuli including hyperglycemia, hypertension, hyperlipidemia, infectious agents, obesity, modified lipopr-

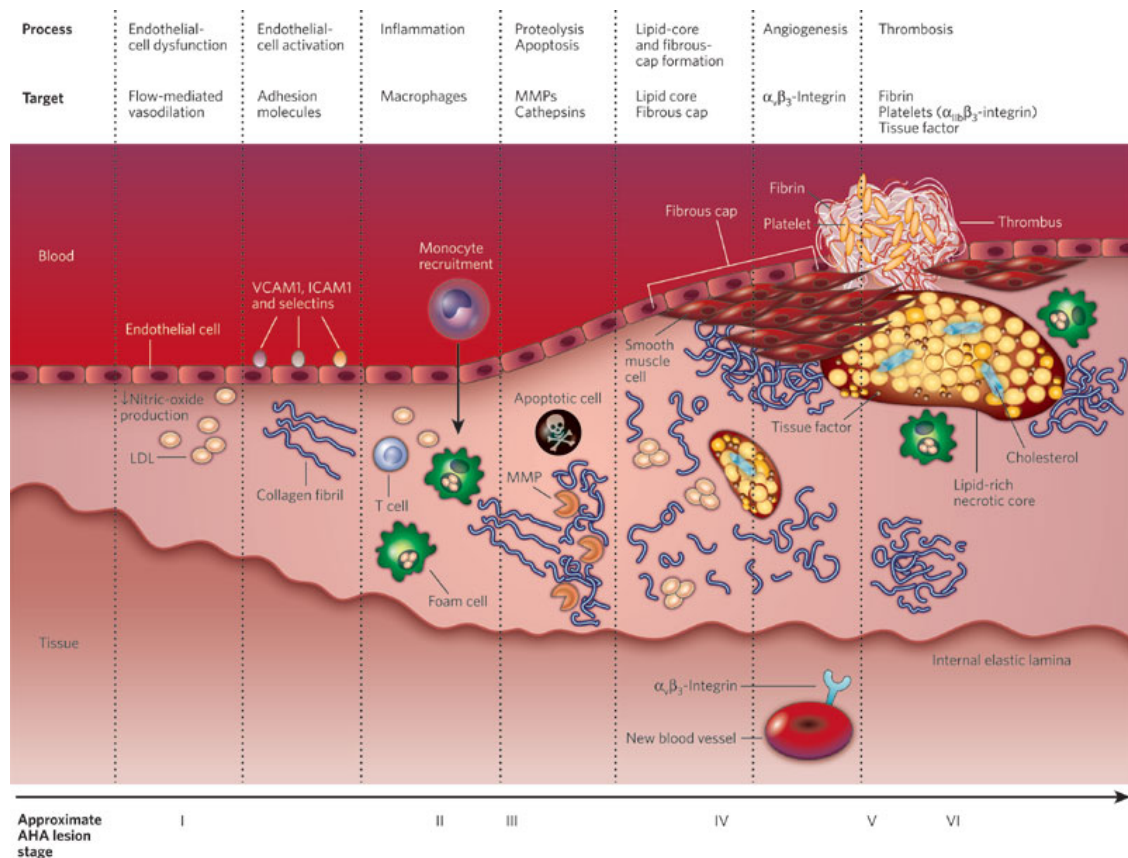


Figure 4. The development and progression of atherosclerotic plaques. © 2008 J Sanz and ZA Fayad. *Imaging of atherosclerotic cardiovascular disease. Nature.* Image used with permission from Nature Publishing Group on July 2, 2012 [45].

oteins, homocysteine, nicotine and free radicals caused by cigarette smoking, altered changes in arterial blood flow shear stress and even normal spontaneous metabolic damage [43, 44, 46-48]. This initial damage induces a loss of basal endothelial homeostasis causing endothelial dysfunction [49]. The resulting increase in endothelial permeability permits the accumulation of low-density lipoprotein (LDL) and cellular debris within the tunica intima of the vessel wall, eventually leading to endothelial activation. Once activated, ECs produce an array of chemoattractant cytokines such as monocyte chemoattractant protein-1, macrophage colony-stimulating factor, interferon- γ , platelet-endothelial cell adhesion molecule-1, interleukin (IL)-1, IL-6 and tumor necrosis factor- α (TNF- α), creating a pro-inflammatory environment that attracts circulating monocytes and T lymphocytes [50-52]. Normally, ECs do not express molecules that facilitate the adhesion of circulating leukocytes. However, activated ECs express vascular cell adhesion molecules such as vascular cell adhesion molecules (VCAM), intercellular adhesion molecules (ICAM), E-selectin, and P-selectin, which mediate leukocyte adhesion and infiltration [50, 52]. Prolonged endothelial dysfunction can lead to cell apoptosis, which ultimately denudes the vessel wall exposing it to the circulating substances within blood [53, 54].

3.2 *Monocytes and Macrophage*

After the mobilization and infiltration of monocytes into the tunica intima, endothelial derived cytokines drive the differentiation of monocytes into macrophages, which use pattern recognition receptors to sequester LDL, modified LDL, free cholesterol (FC) and cholesteryl esters (CE) [55]. Accumulation of cholesterol in macrophages causes foam cell formation, cell immobilization, apoptosis and secondary necrosis.

Macrophage immobilization is associated with increased levels of Rac-GTP, the active form of Rac1, and reduced levels of RhoA. Rac1 induces polymerization of actin that forms both lamellipodia and membrane ruffles while RhoA is associated with actin filament organization and promotion of focal adhesions. Normally, macrophages and monocytes utilize Rac1 and RhoA to facilitate migration through cell motility cycling [56]. The translocation of Rac1 to the plasma membrane facilitates its activation. Cholesterol-rich domains, created during sterol loading, within the inner leaflet of the plasma membrane maintains Rac-GTP in the membrane. The loss of proper cell polarization, cell spreading, and plasma membrane ruffling caused by increased Rac1 activity abrogates forward movement of the cell [57-59].

As immobilized macrophages accumulate within the tunica intima they become apoptotic or necrotic and release proteolytic enzymes, predominantly matrix metalloproteinases (MMP: a family of enzymes that binds zinc at their active sites), that are involved in extracellular matrix and fibrous cap degradation and plaque disruption [8].

3.3 *Vascular smooth muscle cells*

Together, activated macrophage and endothelial cells continue to produce pro-inflammatory cytokines and growth factors that promote the transition of VSMCs from a quiescent, contractile phenotype to an active, proliferative synthetic phenotype that deposits extracellular matrix at the site of injury to form a fibrous cap [60-63].

The proliferation of VSMCs indicates the onset of arterial intimal thickening and has been well characterized. A conglomerate of growth factors, such as epidermal growth factor, platelet-derived growth factor, and fibroblast growth factor as well lipid

accumulation, all cooperate to induce proliferation [64-66]. Upon stimulation, VSMCs transform from the mature, contractile phenotype to the de-differentiated, synthetic phenotype through a reactivation of embryonic gene expression that causes VSMCs to re-enter the cell cycle [67].

Phenotypic change has been correlated to changes in the expression and distribution of structural proteins as well as cell-cell and cell-substrate interactions. For instance, the transition from contractile to synthetic phenotypes is associated with a decrease in VSMC contractile proteins (such as α -smooth muscle actin, smooth muscle-myosin heavy chain, and calponin), marker genes (such as h-caldesmon and SM22- α) and an increase in β -non-muscle actin [68-70]. In addition, the localization of proteins, such as β -non-muscle actin, α -actinin, and vimentin, modifies to increase VSMC motility [70]. Simultaneously, phenotypic change increases collagen synthesis approximately ~25-30 fold as well as augments the relative proportions of collagen I and III synthesis. Akin to macrophages, VSMC can become foam cells by the accumulation of intercellular lipids. Phenotypic change from contractile to synthetic is associated with the increased synthesis of elastin, which is associated with increasing cholesteryl-ester synthesis, lipid binding and foam cell transformation [71-73]. Finally, morphological changes associated with phenotypic transition include decreases in cell adhesive proteins, such as vinculin, and changes in VSMC's interaction with the extracellular matrix.

Phenotypic modulation of VSMCs not only causes changes in cells' protein distribution and synthesis but also affects cell responsiveness to various substances. For instance, the synthetic phenotype has reduced sensitivity to common vasoactive substances such endothelin-1, angiotensin-II, histamine and NE. Synthetic VSMCs can

also perpetuate oxidative stress during atherosclerotic plaque progression through increased inducible NO synthase synthesis [74, 75]. Despite large increase in NO production, VSMCs with the synthetic phenotype lack the β -subunit of guanylyl cyclase and are less sensitive to NO [76]. Moreover, de-differentiation of VSMC decreases the affinity of HDL and decreases apoA mediated cholesterol efflux, thus contributing to the early events of foam cell transformation [73].

Although VSMC proliferation is a well known component of atherosclerosis, less acknowledged is the importance of apoptosis. During atherosclerosis, apoptosis is considered a main process contributing to both plaque development and destabilization [77, 78]. For instance, loss of VSMC through apoptosis significantly decreases plaque stability through reduction of type I collagen production. Moreover, apoptotic VSMCs release to the diseased intima its intracellular contents including calcifying matrix vesicles, proteases, and thrombotic factors, thus contributing to plaque calcification, destabilization and thrombogenicity [79, 80].

4. *Regression of Atherosclerosis*

During the past few decades, an improved understanding of atherosclerotic pathophysiology has enabled researchers to consider the possibility of inducing plaque regression in addition to the more conventional therapeutic goal of reducing plaque progression. Traditionally, atherosclerosis was viewed as an inevitable unidirectional process that begins in childhood and manifests during adulthood [8]. However, during the 1980s, Badimon et al. [7] showed that plaque development is not simply a permanent process associated with age, but rather a dynamic process that can be slowed, stopped or

reversed. In spite of the evidence supporting the existence of plaque regression, the concept of 'plaque regression' was resisted for decades. Critics of regression asserted that the physical attributes of an atherosclerotic plaque, including calcification, necrosis and fibrosis, indicated stability, and the molecular processes associated with plaques such as oxidative damage, cell proliferation and transformation implied that regression would be very difficult or impossible to achieve. Furthermore, majority of animal studies that attempted to induce regression in advanced plaques had failed [44, 81-84]. However, the ability of even advanced lesions to regress on robust reductions in LDL levels and increases in high-density lipoprotein (HDL) levels has established plaque regression as a well-accepted process [10, 85]. Atherosclerosis is now known as a highly dynamic process in which plaques are able to either progress or regress, depending on the surrounding milieu [86].

In an ideal setting, plaque regression would be the return of the arterial wall to its initial healthy and fully functional state. However, with the exception of fatty streaks, even under ideal conditions, the complete reversal of plaque formation never occurs [86]. We point out that plaque regression is unique and not simply plaque progression in reverse. Rather, the mechanisms of plaque regression are distinct and are composed of three important processes: the reduction or clearance of necrotic and extracellular material from the tunica intima; endothelial repair, regeneration and return to homeostasis; and the cessation of smooth muscle cell proliferation (Figure 5). The rapid development of diagnostic imaging has created technological modalities and indexes for the assessment of the arterial wall and measurement of atherosclerotic plaques. Common indexes such as luminal area, plaque volume or intimal medial thickness (IMT) estimate

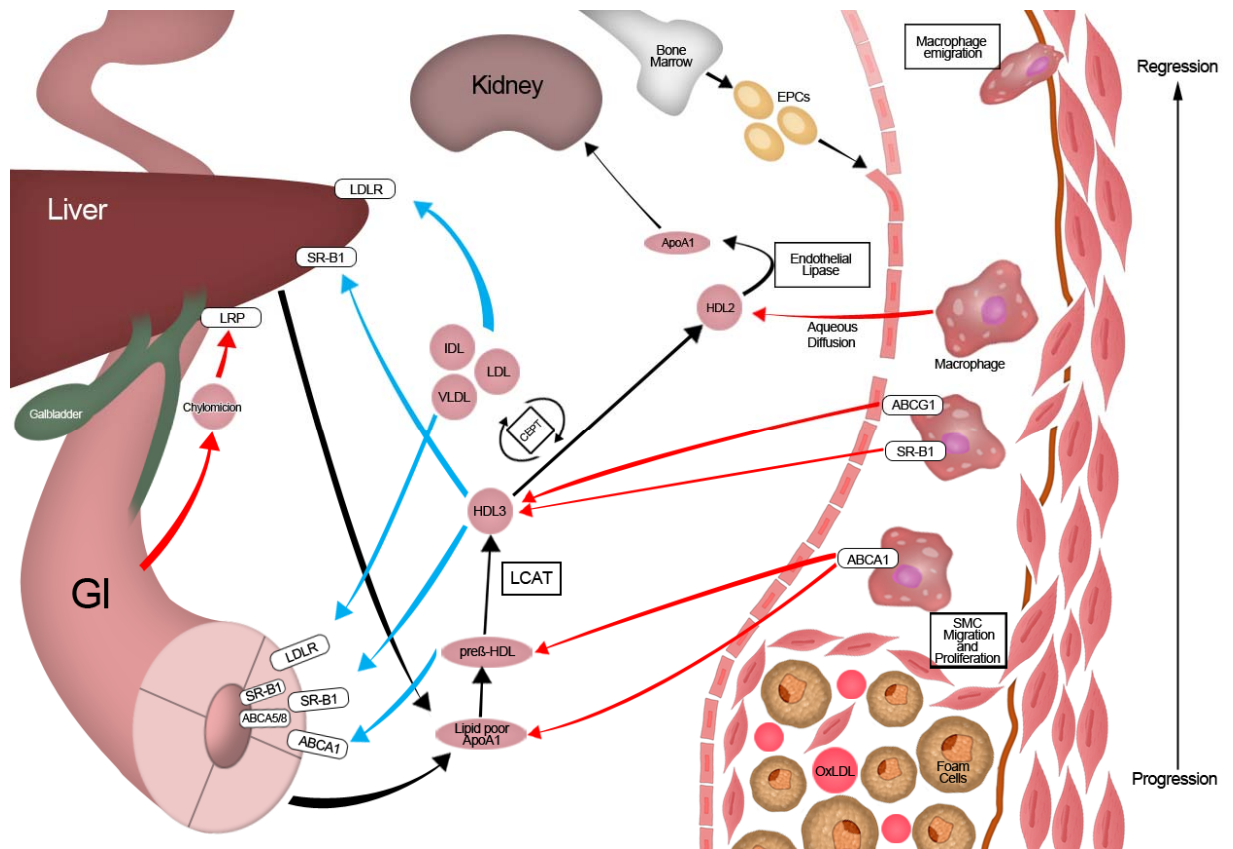


Figure 5. The integrated mechanism of atherosclerotic plaque regression. © 2011 A Francis and GN Pierce. *An integrated approach for the mechanisms responsible for atherosclerotic plaque regression. This information was originally published in Exp Clin Cardiol 2011;16(3):77-86. Permission was granted from Pulsus Group Inc. on July 3, 2012 [9].*

plaque regression. In addition, novel modalities can crudely characterize plaque composition and fibrous cap thickness. However, all diagnostic imaging modalities are currently unable to capture the true complexity of atherosclerosis.

4.1 HDL and regression

Although the mechanisms of regression are beginning to be understood, there has been considerable progress in isolating the important players in regression and understanding their biological roles. Attention, however, has been focused on reverse cholesterol transport (RCT) and its major player, HDL.

According to the generally accepted definition of RCT, the first step of RCT is the efflux of extrahepatic cholesterol from intracellular cholesterol pools, primarily focusing on the removal of cholesterol from macrophages. Alternatively, critics of this view attest that assembly of HDL particles and their subsequent secretion into the circulation is the initial step of this process [87-89]. However, for a thorough overview of HDL's involvement in RCT, the production and release of HDL must be evaluated. Despite the disagreement regarding the initial steps in RCT, it is accepted that the acquisition of cholesterol from peripheral stores by HDL is the major mechanism for promoting atherosclerotic plaque regression.

The production of all HDL subclasses begins with the synthesis of apoAI and apoAII. These proteins are primarily generated in the liver and secondarily by the small intestine [89, 90]. Both hepatocytes and enterocytes can secrete lipid-poor apoAI and AII into the circulation, where it acquires phospholipids and cholesterol to form pre β -HDL [91-93]. Regardless, the majority of apoA produced in the intestine is packaged into chylomicrons, which are eventually internalized by the liver. In contrast, hepatocyte apoA secretion into plasma is more complex. Similar to chylomicrons, very low-density lipoprotein (VLDL) – another triglyceride-rich lipoprotein – can lose cholesterol and phospholipids while losing or gaining apolipoproteins during lipolysis, which generates pre β -HDL. VLDL can also be amalgamated into HDL₂ and HDL₃ [94]. Finally, hepatocytes can synthesize and secrete both apoA and pre β -HDL directly into the circulation. In spite of the different modes of release, once in the circulation, pre β -HDL can be used for RCT.

The first and rate-limiting steps of RCT is cholesterol efflux from macrophages, and the peripheral tissue to the HDL particle [95]. There are currently four proposed mechanisms of cholesterol efflux into lipid-poor apoAI and the various HDL particles: aqueous diffusion, scavenger receptor class B type 1 (SR-BI)-mediated efflux, adenosine triphosphate (ATP)-binding cassette (ABC) transporter A1-mediated efflux and ABCG1-mediated efflux [87, 88].

First, cholesterol efflux can occur passively in adherence to its concentration gradient. As a result, the passive diffusion pathway is bidirectional, and the rate-limiting step of this process is dependent on the ratios and interactions between the FC in the donor cells and the phospholipids within the plasma membrane of the acceptor molecule – the HDL particle [95, 96]. The kinetics of this interaction is accelerated by higher proportions of unsaturated phospholipids and decreases in the sphingomyelin content of the plasma membrane [97, 98]. Since cholesterol efflux by this pathway is not affected by HDL particle size, all HDL subclasses are equal in their ability to accept cholesterol through aqueous diffusion [99].

In contrast to aqueous diffusion, the remaining three cholesterol efflux pathways are mediated by protein-protein interactions. Cholesterol efflux can be mediated by the ATP-independent, passive SR-BI pathway. Cholesterol transport is initiated by the binding of HDL to SR-BI, which subsequently forms a hydrophobic channel allowing efflux to occur [100]. SR-BI promotes efflux exclusively to larger, more mature HDL particles such as HDL₂ and HDL₃, which can further increase the SR-BI-mediated selective uptake of cholesterol if they contain apoAII [101]. Because this process is bidirectional, HDL may be a donor and an acceptor of cholesterol. Thus, its role in

aiding RCT in macrophages may not be as important as the remaining energy-dependent cholesterol efflux pathways [102].

Cholesterol and phospholipid efflux to apoA1 is promoted by the interaction of ABCA1 with pre β -HDL or with lipid-poor apoA1 [103]. The activation of ABCA1 induces the removal of cholesterol from the plasma membrane of the donor cell and can also cause efflux from intracellular cholesterol pools [104, 105]. The rate of lipid efflux is dependent on the concentration of activated ABCA1 and on the amount of unsaturated phospholipids in the donor cell [106]. In addition, the concentration of ABCA1 in the plasma membrane is regulated by the interaction of apoA1 and ABCA1, which prevents its intracellular degradation [107].

Another member of the ABC family of transporters, ABCG1, is the last major efflux pathway in RCT. ABCG1, which is involved with regulating intracellular cholesterol homeostasis, interacts with all subclasses of HDL [108-110]. It mediates cholesterol efflux through the reorganization of existing cholesterol pools, thereby increasing cholesterol diffusion to HDL particles. Although all HDL subclasses can mediate efflux through the ABCG1 pathway, larger HDL particles are more effective acceptors, as in the SR-BI efflux pathway [111].

Following the removal of cholesterol from peripheral sites, HDL undergoes further maturation characterized by an increase in particle size due to the progressive accumulation of cholesterol [112]. In circulation, cholesterol becomes esterified to pre β -HDL through an apoA1-mediated reaction with lecithin:cholesterol acyltransferase, converting lecithin and cholesterol into lysolecithin and CE [113]. Because CEs are hydrophobic, they can move to the developing core of the HDL particle, which causes it

to enlarge and become spherical. This ultimately transforms pre β -HDL into HDL₃ or HDL₂. This process not only increases the surface area for FC acceptance, it also helps maintain the FC gradient [102, 114]. Once a mature HDL particle is synthesized, it has three possible fates. First, lipolytic enzymes such as lipoprotein, hepatic and endothelial lipases can hydrolyze triglycerides and phospholipids causing apoAI to dissociate from HDL, which facilitates the clearing of apoAI from circulation by the kidneys and the conversion of mature HDL particles back into nascent pre β -HDL [115-117]. Second, constituents of mature HDL particles may be transported to other HDL subclasses or other lipoproteins. For example, certain plasma lipid transfer proteins can transfer triacylglycerol and phospholipids amongst lipoproteins [117]. CETP transfers the majority of CEs to apoB-containing lipoproteins, such as VLDL and LDL, which produce small cholesterol ester-depleted HDL particles that are either cleared by the kidneys or incorporated into another HDL particle to form a large mature HDL particle [118]. ApoB-containing lipoproteins can be removed from the circulation by hepatic LDL receptors and catabolized by the liver, and hepatic SR-BI can also bind LDL and VLDL. Thus, the CETP pathway is believed to be an important route of RCT [119-122]. However, it is also likely that cholesterol transferred to LDL or VLDL can be re-deposited to peripheral tissue. Third, cholesterol can be returned to the liver through the clearance of HDL from the circulation. SR-BI, located on the surface of liver or adrenal cells, recognizes circulating HDL particles and facilitates the unloading of their FC and cholesterol esters [123]. This process does not involve the internalization and degradation of the HDL particle. Thus, removal of cholesterol and CEs produces nascent HDL particles that have the potential to be recycled back into the RCT pathway [118].

As opposed to HDL efflux from macrophages, SR-BI is the most abundant receptor on hepatocytes, thus making this pathway an important mode of cholesterol influx. An alternative method of influx involves the acquisition of apoE enabling HDL to deliver cholesterol to hepatic tissue through its interaction with LDL receptors, which mediates the internalization of HDL particles and their subsequent catabolism [124]. Novel routes to HDL holoparticle uptake by hepatocytes, such as HDL internalization by the receptor P2Y13, continue to be identified [125].

Cholesterol within hepatocytes must be secreted into the intestinal lumen to complete the RCT pathway. The rate of cholesterol excretion is dependent on several transporters on its apical membrane. Cholesterol efflux into bile is dependent on both SR-BI and the ABC family of transporters. The rate of cholesterol efflux is primarily controlled by ABCG5 and ABCG8 transporters [126, 127]. Although SR-BI can also facilitate cholesterol secretion into bile, it serves only a minor role on the apical membrane, being secondary to the ABC transporters [128, 129].

The hepatobiliary route of HDL-C removal is the major mode of cholesterol excretion. However, Cheng and Stanley [130] suggested that there is an additional route to fecal cholesterol loss through a non-biliary route. Non-biliary sterol loss is thought to be regulated by the liver. Under conditions in which the hepatic cholesterol load exceeds a manageable amount, cholesterol is repackaged into plasma lipoproteins that are bound for secretion by the intestines [131]. Although the mechanism of the nonbiliary fecal pathway is not well understood, many aspects are shared with the hepatobiliary route of sterol loss. It has been suggested that lipoproteins responsible for the transport of cholesterol to the intestine are either apoE-rich HDL or apoB-containing lipoproteins

[132, 133]. It is likely that most lipoproteins are able to facilitate this process because enterocytes express SB-RI on their basolateral membrane [112]. Similar to hepatocytes, enterocytes express ABCG5/G8 as well as SR-BI on their apical membranes, allowing the efflux of cholesterol from the enterocyte to the intestinal lumen [134].

4.2 *Endothelial cells and regression*

To achieve atherosclerotic plaque regression, dysfunctional ECs must be returned to basal homeostasis and dead cells need to be replaced. Currently, there are three mechanisms that can result in endothelial cell replacement and reestablish the homeostatic function of the endothelium: circulating endothelial progenitor cells, local endothelial cell proliferation and migration, and abrogation of endothelial apoptosis.

The term ‘stem cell’ or ‘progenitor cell’ refers to immature cells that have the ability to self-renew and differentiate into a variety of cell types. Thus, these cells have the potential to restore the function of damaged tissues [135, 136]. Endothelial progenitor cells (EPCs), similar to all progenitor cells, are lineage specific, and comprise a highly heterogeneous population of cells capable of differentiating exclusively into ECs [137]. The majority of progenitor cells mature from hematopoietic stem cells. These stem cells are mainly isolated from bone marrow, peripheral blood and umbilical cord, but can be obtained from the spleen, intestine, liver, adipose tissue and adventitia [138]. Regardless of their source, all hematopoietic stem cells are CD34⁺ and CD133⁺. Hematopoietic stem cells can also produce non-erythroid myeloid and granulocyte-macrophage lineages, as well as EPCs. Therefore, EPCs are characterized by the co-expression of both hematopoietic stem cell markers and endothelial markers such as

vascular endothelial growth factor receptor-2, CD31, endothelial NO synthase, and vascular endothelial cadherin [138-142].

To obtain any benefit from non-tissue resident or exogenously administered EPCs, they must be targeted to the site of injury. This process is orchestrated by resident cells of the injured area [142]. During atherosclerosis, various cell types within the plaque are capable of mobilizing and homing EPCs to denudated vessels. First, EPCs are released from their source upon stimulation from molecular signals produced by immune cells within the plaque. Activated M2 type macrophages promote vessel healing through the secretion of granulocyte colony-stimulating factor (G-CSF) [143]. G-CSF facilitates the release of EPCs into circulation. In addition, cytokine-mediated release of proteases such as elastase, cathepsin G and MMP-9 discharges EPCs by cleaving the adhesive interaction between EPCs and stromal cells [142, 144]. The released EPCs subsequently home to the injured area. An important homing signal is the chemokine stromal cell-derived factor-1 (SDF-1). Under normal conditions, the bone marrow and many other tissues constitutively express SDF-1. The bone marrow, however, produces a gradient that favours the retention of EPCs. During conditions of ischemia, inflammation and hypoxia, this gradient is reversed by the expression of hypoxia-inducible factor-1, which upregulates SDF-1 in injured tissues [145-148]. In addition, NO, estrogen, HDL, vascular endothelial growth factor and erythropoietin contribute to the increase in the plasma titre of EPCs and their recruitment to the site of injury by augmenting the phosphatidylinositol-3-phosphate (PIP3)/Akt pathway [149-151]. Once in the damaged area, cell adhesion molecules, such as P/E-selectin and ICAM-1, mediate the binding of EPCs to the injured endothelium [152]. In severely damaged vessels, exposed matrix

proteins activate platelets that adhere to the denuded area. Platelet activation causes microthrombi formation and the expression of SDF-1, targeting EPCs to the damaged endothelium. After EPC attachment, they differentiate into ECs under the influence of the laminar shear stress produced during blood flow [136, 153].

Endothelial migration and proliferation is promoted by HDL. Endothelial migration may be stimulated by the interaction between SR-BI and HDL [154]. Alternatively, sphingosine-1-phosphate within HDL can induce endothelial cell migration by activating Ras/Raf1-dependent ERK [154, 155]. The induction of EC proliferation by HDL is mediated by down-regulating ADAMTS-1, by activating the protein kinase C pathway and by increasing intracellular Ca^{2+} levels through phospholipase C activation [156-158].

As atherosclerosis persists, ECs become apoptotic, leading to the denudation of the vessel wall. Apoptosis is initiated by the activation of the death receptor and mitochondrial-mediated apoptotic pathways. HDL also may have a role in preventing EC apoptosis and promoting endothelial repair [159]. HDL can directly or indirectly inhibit endothelial apoptosis either by decreasing the levels of TNF- α , oxidized LDL and growth factors, or by inhibiting the apoptotic pathways. These anti-apoptotic properties are mediated by constituents within HDL. ApoA1, an important protein of the RCT pathways, diminishes oxidized LDL and TNF- α induced apoptosis [160, 161]. Lysosphingolipids within HDL, such as sphingosylphosphorylcholine and lysosulfatide, can inhibit growth factor-induced apoptosis as well as directly interfere with apoptotic signaling within ECs by activating the Akt signaling pathways [155, 162-164].

4.3 *Macrophage and regression*

During regression, cholesterol removal is accompanied by the disappearance of macrophage and foam cells. This is one of the first noticeable indications of plaque regression [86]. It was initially believed that the disappearance of monocytes and macrophages from atherosclerotic plaques was only caused by apoptosis, lysis in situ and phagocytosis by new macrophages. However, it is now evident that macrophages within atherosclerotic lesions can also regain motility and migrate to regional lymph nodes when the local environment improves.

The migration of macrophage and foam cells from atherosclerotic plaques is complex and is controlled by plaque dynamics. Improvements in the plaque milieu causes immobilized macrophage to migrate by activating the expression of dendritic cell markers [165, 166] such as the chemokine (C-C motif) receptor 7 (CCR7), an essential requirement for dendrite cell migration [167]. CCR7 control of immune cell emigration is mediated by the activation and upregulation of the liver X receptor [85]. The downstream target of the liver X receptor, ABCA1, is also unregulated [166]. Macrophage/foam cell migration and morphological transformation can be, in part, facilitated by ABCA1-induced cholesterol redistribution. ABCA1 may decrease membrane cholesterol pools releasing Rac-GTP from the plasma membrane [168, 169]. ABCG1 and LDL receptor-related protein 1 may also play a small role in macrophage migration from atherosclerotic plaques [167, 170].

4.4 *Vascular smooth muscle cells and regression*

Despite the large amount of evidence linking VSMC to atherosclerotic plaque progression, little is known about their involvement in atherosclerotic plaque regression.

Nevertheless, regression can be induced by the removal of lipids from sterol-loaded VSMC and by the cessation of VSMC proliferation, which ultimately reverses the pathological phenotypic. Sterol unloading from VSMCs shares many similarities with macrophage sterol removal. SR-BI, a mediator of cholesterol efflux in macrophages and influx in hepatocytes, is also expressed in VSMCs [171]. Although synthetic VSMCs have a reduced affinity for NO, NO is still capable of inhibiting smooth muscle cell proliferation in culture. Dietary supplementation with L-arginine, the precursor of NO, has also been shown to induce plaque regression in cholesterol-fed rabbits [172]. Bioactive fatty acids such as prostacyclin I1 and prostaglandin E2 maintain the contractile phenotype of VSMCs and play a major role in reducing migration and proliferation of VSMCs through activation of peroxisome proliferator-activated receptors (PPARs). Activation of both PPAR- γ and PPAR- α leads to the suppression of pro-inflammatory cytokines by inhibiting the activity of nuclear factor κ B (NF- κ B) [173, 174]. In addition, PPAR- α inhibits proliferation through p16/pRb/E2F-mediated suppression of telomerase activity. PPAR- δ activity inhibits VSMC migration and proliferation by blocking the cell cycle [175-177]. Finally, PPAR activation regulates lipid metabolism and inhibits foam cell formation by augmenting the expression of scavenger receptors [178].

5. *Nutrition and Cardiovascular disease*

5.1 *Nutrition and regression*

Although there is a copious amount of data in the literature concerning the mechanisms of regression, it is important to translate this information into a clinical

setting and target/monitor the various proponents of regression. A variety of pharmacological treatments cause robust alterations in lipoprotein levels, such as LDL and HDL, and ameliorate cardiovascular risk factors, such as hypertensive and hyperglycemia. These therapies have the ability to induce atherosclerotic plaque regression (Tables 1-4). However, many of these pharmaceuticals have deleterious side effects, which ultimately limit their long-term use. Thus, considerable attention has been directed towards lifestyle modifications, specifically nutritional interventions, and their ability to induce atherosclerotic plaque regression.

Hippocrates (460 B. C. - 370 B.C), the father of western medicine, stated “let food be thy medicine and medicine by thy food” [179]. The ability of nutritional interventions to prevent or reduce the incidence of diseases, including CVD, has been known for centuries. However, there is very little evidence implicating nutrition with the regression of atherosclerotic plaques. Currently, only the Mediterranean diet has shown the greatest promise in its ability to cause the regression of plaques.

The Mediterranean diet is characterized by high proportions of monounsaturated fatty acid (MUFA) and PUFA intake, with a daily consumption of vegetables, fruits, whole grains and low-fat dairy products, fish, poultry, nuts and legumes accompanied with moderate alcohol intake and low intake of red meats, processed products and saturated fat-rich foods [180]. This dietary pattern has been associated with both cardio-protective and anti-atherosclerotic effects. Carotid IMT, a measurement used to assess changes in plaque thickness, has an inverse relationship with maintenance on the Mediterranean diet [181]. In addition, a recent meta-analysis of 50 randomized controlled trials and observational studies has shown that a 30% risk reduction of

Table 1. LDL-lowering treatments (Statins) able to induce atherosclerotic plaque regression as assessed by different imaging modalities

Study	Study Design	N	Medication	Follow up (months)	Endpoint	Results	P-value (from baseline)
<i>B-mode Ultrasound</i>							
ASAP [183]	RCT	325	Atrovostatin (80 mg/day) n=160 Simvastatin (40 mg/day) n=165	24	↓ in carotid IMT	Atrovostatin decreased carotid IMT	P < 0.0001
ARBITER [184]	RCT	161	Atrovostatin (80 mg/day) n=79 Pravastatin (40 mg/day) n=82	12	↓ in carotid IMT	Atrovostatin decreased carotid IMT	P = 0.03
METEOR [185]	RCT	876	Rosuvastatin (40 mg/day) n = 624 Placebo n=252	24	↓ in carotid IMT	Rosuvastatin decreased carotid IMT	P<0.001
<i>Grey scale Intravascular Ultrasound</i>							
Petroni et al. [186]	RCT	71	Simvastatin (20 mg/day) No treatment	12	↓ in coronary PPV ↓ in coronary NSP	Statin therapy decreased PPV and NSP	P<0.05
JAPAN-ACS [187]	RCT	252	Pitavastatin (4mg/day) Atrovostatin (20 mg/day)	12	↓ in coronary PV	Both treatments decreased PV	P<0.001
ASTEROID [188]	RCT	349	Rosuvastatin (40 mg/day)	24	↓ in coronary PAV	Statin therapy decreased PAV	P<0.001
COSMOS [189]	RCT	126	Rosuvastatin (2.5 mg/day increased to <20 mg/day at 4 week intervals)	18	↓ in coronary PAV	Statin therapy decreased PAV	P<0.0001
SATURN [190]	RCT	1039	Atrovostatin (80 mg/day) n=519 Rosuvastatin (40 mg/day) n = 520	24	↓ in coronary PAV and TAV	Both treatments decrease PVA and TVA	P<0.001

Randomized controlled trial = RCT; Observational study = OS; Persistent plaque volume = PPS; nonstented plaque volume = NSPV; Perspective single blinded= PSB; Percent atheroma volume = PAV; Plaque volume = PV; vessel wall area = VWA; vessel wall volume = VWV; Luminal area = LA; Plaque area = PA; Ultrasmall superparamagnetic iron oxide = USPIO

Table 1 (continued). LDL-lowering treatments (Statins) able to induce atherosclerotic plaque regression as assessed by different imaging modalities

Study	Study Design	N	Medication	Follow up (months)	Endpoint	Results	P-value (from baseline)
<i>Virtual Histology Intravascular Ultrasound</i>							
Kawasaki et al. [191]	RCT	52	Pravastatin (20 mg/day) n=17 Atrovastatin (20 mg/day) n=18 Diet (control) n=17	6	Changes in plaque composition and volume of coronary arteries	no change in PV and stenosis area and diameter Increase in fibrous volume and reduction in lipid volume vs control	P<0.05
Yokoyama et al. [192]	RCT	50	Atrovastatin (10 mg/day) n=25 Diet (Control) n=25	6	Changes in plaque composition and volume of coronary arteries	Statin therapy decreased plaque volume and changes integrated backscatter-defined plaque composition	P<0.0001
Miyagi et al. [193]	OS	100	A Statin n=44 No Statin n=56	≥ 6	Changes in plaque composition and volume of coronary arteries	Increase in fibrous volume and reduction in lipid volume in statin group without changes in PV	P =0.003 lipid volume P <0.001 fibrous volume
Hong et al. [194]	RCT	100	Simvastatin (20 mg/day) n = 50 Rosuvastatin (10 mg/day) n = 50	12	Changes in plaque composition and volume of coronary arteries	Decrease in necrotic core volume and increase in fibrofatty volume in Rosuvastatin	P < 0.05
Nasu et al. [195]	RCT	80	Fluvastatin (60 mg/day) n=40 Control n = 40	12	Changes in plaque composition and volume of coronary arteries	Decrease in fibrofatty, necrotic plaque volume and increase in fibrous volume	P <0.0001 P = 0.004 necrotic core
Toi et al. [196]	RCT	160	Pitavastatin (2 mg/day) n = 80 Atrovastatin (10 mg/day) n = 80	2-3 (weeks)	Changes in plaque composition and volume of coronary arteries	Plaque and fibrofatty volume index decreased with Pitavastatin treatment	P<0.05
Shin et al. [197]	RCT	48	Simvastatin (20 mg/day) n = 24 Rosuvastatin (10 mg/day) n = 24	12	Changes in plaque composition and volume of coronary arteries	Dense calcium increased while fibrous and fibrofatty volumes decreased with increases in necrotic core in the simvastatin arm	P <0.001
Nozue et al. [198]	RCT	119	Pitavastatin (4mg/day) n = 58 Pravastatin (20 mg/day) n= 61	8	Changes in plaque composition and volume of coronary arteries	Both treatments decreased fibrofatty volumes and increased	P≤0.005

Table 1 (continued). LDL-lowering treatments (Statins) able to induce atherosclerotic plaque regression as assessed by different imaging modalities

Study	Study Design	N	Medication	Follow up (months)	Endpoint	Results	P-value (from baseline)
<i>Magnetic Resonance Imaging</i>							
Corti et al. [199]	RCT	51	Simvastatin (20 mg/day) n = 29 Simvastatin (80 mg/day) n = 22	18.1	↓ in VWA in the aorta and carotid arteries	Decrease in both carotid and aortic VWA with statin treatment.	P<0.001
Corti et al. [200]	?	21	Simvastatin (? mg/day)	24	↓ in VWA and VWT in the aorta and carotid arteries	Decrease in both carotid and aortic VWA and VWT	P <0.01
Lima et al. [201]	RCT	27	Simvastatin (20 to 80 mg/day)	6	↓ in LA, PV, PA in the thoracic aorta	Decreases in plaque volume and area	P <0.05
Yonemura et al. [202]	RCT	36	Atorvastatin (5 mg/day) n = 18 Atorvastatin (20 mg/day) n= 18	24	↓ in VWA and VWT in the thoracic and abdominal aorta	20 mg/dl of Atorvastatin decreased thoracic VWA and VWT (mostly seen at 1 year)	P < 0.001
ORION [203]	RCT	33	Rosuvastatin (5 mg/day) n = 13/ n= 8 Rosuvastatin (40 mg/day) n= 20/ n= 10 (Plaque morphology/plaque composition)	24	↓ in carotid VWV and Changes in plaque composition	Lipid rich necrotic core decreased from baseline in all patients	P < 0.005
ATHEROMA [204]	RCT	40	Atorvastatin (10 mg/day) n = 20 Atorvastatin (10 mg/day) n = 20	2	Changes in plaque inflammation	High doses statin reduced MRI-USPIO defined inflammation	P < 0.0001
CPC [205]	RCT	33	Atorvastatin (10 to 80 mg/day) Atorvastatin (same doses) and ER-niasin (2g/day) Atorvastatin (same doses), ER-niasin (same dose), and colesvelam (3.8g/day)	36	Changes in carotid plaque lipid content, burden and composition	Grouped together lipid treatment depletes plaque lipid volume and decreases vessel wall volume and increases plaque fibrous components	P <0.0001

Table 1 (continued). LDL-lowering treatments (Statins) able to induce atherosclerotic plaque regression as assessed by different imaging modalities

Study	Study Design	N	Medication	Follow up (months)	Endpoint	Results	P-value (from baseline)
<i>Optical Coherence Tomography</i>							
Hattori et al. [206]	RCT	42	Pitavastatin (4mg/day) n=26 Diet (control) n=16	9	Changes in plaque composition, volume and fibrous cap dimensions of coronary arteries	Statin therapy reduced plaque and lipid volume index and increase fibrous volume index and cap thickness	P<0.05
Takarada et al. [207]	OS	40	A Statin n = 23 Control n =17	9	Changes fibrous cap dimensions of coronary arteries	Statin treatment increased fibrous cap thickness	P<0.001
<i>Computed Tomography</i>							
Inoue et al. [208]	OS	32	Fluvastatin (20 mg/day) n = 24 Control n = 8	6	Changes in Plaque morphology	Statin treatment decreased plaque and necrotic core volume	P <0.01

Table 2. LDL lowering treatments (Ezetimibe) able to induce atherosclerotic plaque regression as assessed by different imaging modalities

Study	Study Design	N	Medication	Follow up (months)	Endpoint	Results	P-value (from baseline)
<i>B-mode Ultrasound</i>							
Bogiatzi et al. [209]	OS	231	Combination therapy without Ezetimibe (2 years) Combination therapy with Ezetimibe (an addition 2 years)	48	↓ in carotid TPA	Addition of Ezetimibe decreased total plaque area	P < 0.01
<i>Virtual Histology Intravascular Ultrasound</i>							
HEAVEN [210]	RCT	89	Atorvastatin (80 mg/day) and Ezetimibe (10 mg/day)	12	Changes in plaque composition and PV of coronary arteries	Aggressive lipid lowering decreased PVA and combined atherosclerosis regression	P=0.014 (PVA) P = 0.007

Randomized controlled trial = RCT; Observational study = OS; Total plaque area = TPA; Plaque volume

Table 3. HDL-elevating treatments able to induce atherosclerotic plaque regression as assessed by different imaging modalities

Study	Study Design	N	Medication	Follow up (months)	Endpoint	Results	P-value (from baseline)
Niacin							
<i>B-mode ultrasound</i>							
ARBITER 6-HALTS [211]	RCT	315	Statin therapy + ER-Niasin (2g/day) n=154 Statin therapy + Ezetimibe (10 mg/day) n=161	14	↓ in carotid IMT	Niacin therapy decreased IMT	P < 0.001 vs baseline P < 0.016 vs ezetimibe
HDL substitution and mimetics							
<i>Intravascular ultrasound</i>							
Nissen et al. [212]	RCT	47	High and low dose ETC-216 weekly (45 mg/kg; 15 mg/kg) n = 36 Placebo (0.9% normal saline) n = 11	3-5 (weeks)	↓ in TAV	Treatment decreased TAV and plaque volume	P < 0.001
ERASE [213]	RCT	136	Statin + Low dose CSL-111 weekly (40 mg/kg) n = 89 Statin + Placebo (0.9% normal saline) n = 47	4 (weeks)	↓ in PV, PPV and TAV in coronary arteries	CSL-111 decreased PV, PPV and TAV	P < 0.001 (CSL-111 group)

Randomized controlled trial = RCT; Percent plaque volume = PPV; Total atheroma volume = TAV; Plaque volume = PV; Intimal-medial thickness = IMT

Table 4. Other pharmaceuticals able to induce atherosclerotic plaque regression as assessed by different imaging modalities.

Study	Study Design	N	Medication	Follow up (months)	Endpoint	Results	P-value (from baseline)
Anti-hypertensive treatments							
<i>B-mode Ultrasound</i>							
VHAS [214]	RCT	456	Verapamil (240 mg/day) n = 224 Chlorthalidone (25 mg/day) n = 232	48	↓ in carotid IMT	Both treatments decrease IMT (Arteries with and Mmax >1.2 mm)	P < 0.01
MORE [215]	RCT	154	Olmесartan medoxomil (20 mg/day) n = 78 Atenolol (50 mg/day) n = 76	24	↓ in common carotid IMT and PV	Both treatments decreased IMT	P < 0.0001
<i>Intravascular Ultrasound</i>							
Waseda et al. [216]	OS	64	ARB n = 23 No ARB n = 41	7	↓ in coronary VVI, LVI and PVI	Treatment with ARB only decreased PVI	P < 0.01
Anti-diabetic treatments							
<i>Intravascular Ultrasound</i>							
APPROACH [217]	RCT	462	Rosiglitazone (~6 mg/day) n = 233 Glipizude (~12 mg/day) n = 229	18	↓ in TVA and PAV in worst coronary segments	Rosiglitazone decreased TVA _N and both treatments decreased PVA from baseline	P < 0.05 TVA _N P < 0.0001 PVA
PERISCOPE [218]	RCT	367	Pioglitazone (15-45 mg/day) n = 179 Glimepiride (1-4 mg/day) n = 188	18	↓ in coronary PAV and TVA	Pioglitazone decreased TVA and both treatments decreased PVA in most diseased segments	P < 0.001 TVA _N P = 0.001 G PVA P = 0003 P PVA
Antioxidants							
<i>Intravascular Ultrasound</i>							
CART-2 [219]	RCT	465	AGI-1067 (280 mg/?day) n = 183 Placebo n = 49	12	↓ in coronary PV	Treatment decreased plaque volume compared to baseline	P = 0.001

Randomized controlled trial = RCT; Observational study = OS; Percent atheroma volume = PPV; Total atheroma volume = TAV; Plaque volume = PV; Plaque volume index = PVI; vessel volume index = VVI;

metabolic disease can be achieved with maintenance on a Mediterranean diet [180].

The Mediterranean diet has also been implicated in the regression of atherosclerotic plaques. The PREvención con DIeta MEDiterránea (PREDIMED)-Navarra study [182] was a multi-centered controlled trial that compared the 1-year effects of a dietitian-initiated Mediterranean diet enriched with either virgin olive oil or mixed unprocessed nuts on carotid IMT measurements in subjects that were at high risk for CVD. The dietary intervention significantly ($P = 0.04$) reduced IMT from baseline values in patients with the highest initial atherosclerotic burden.

The Mediterranean diet's association with plaque regression was further validated along with low-carbohydrate and low-fat diets in the (dietary intervention randomized control trial) DIRECT-Carotid trial [181]. In this study, 141 patients with CAD or type 2 diabetes mellitus were randomized to one of three nutritional interventions (Mediterranean diet, low carbohydrate diet and low fat diet) to assess their measurable effects on established IMT and vessel wall volume (VWV) measurements using 3-dimensional ultrasound in carotid arteries. Two years of dietary intervention significantly ($P < 0.001$) reduced (4.9%) carotid VWV. There were no differences between the three dietary treatments. Moreover, they found that decreases in systolic blood pressure might have accounted for the regression of carotid VWV.

The success of the Mediterranean diet in decreasing the incidence of CVD as well as the induction of atherosclerotic plaque regression may be attributed to its high PUFA content. PUFAs are well known functional components of marine and plant foods and are have a strong history of CVD prevention.

5.2 *Polyunsaturated fatty acids and cardiovascular disease*

After a series of six expeditions to Greenland starting in 1960, Bang and Dyerberg observed that the Inuit (Eskimo) population had a very low incidence of CAD despite the consumption of extremely high levels of fat from fish and seal/whale blubber [179, 220]. These observations challenged the traditional view that over consumption of fats (energy) leads to poorer cardiovascular outcomes, and showed cardiovascular health is related to the type of fat intake, considering the Inuit's diet was rich in ω -3 PUFA. Bang and Dyerberg's findings in association with Hugh Sinclair's seminal observation on the detrimental effects of essential fatty acid deficiency on CVD, helped solidify the hypothesis that ω -3 fatty acids provided protection against atherosclerosis and have the potential to be a treatment for vascular disease [221, 222].

Anthropological data from the Palaeolithic period (40,000 years ago) showed that humans may have consumed relatively equal amount of ω -3 and ω -6 PUFAs. However, after the agricultural revolution, the ω -3: ω -6 ratio substantially increased from the 1:1 ratio of our predecessors to the very unhealthy western diet ratios of 1:15 to 1:50, caused from a lack of ω -3 PUFA dietary intake and overconsumption of ω -6 PUFAs [221]. The ω -3 PUFAs that showed cardioprotective properties in the Inuit population of Greenland were primarily, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the main component of marine fish oils [17]. Various clinical studies evaluating the beneficial effect of fish oil supplementation have shown the effectiveness of this treatment on CVD. For example, large-scale clinical trials such as The Diet and Reinfarction Trial (DART), The Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI)-Prevenzione, and the Japan EPA Lipid Intervention study (JELIS) all have shown EPA

and DHA supplementation, simultaneously or separately, causes significant reductions in mortality and relative risk in patients that are at high risk for coronary artery disease (CAD) [223-225]. In addition, fish oil supplementation can significantly reduce primary endpoints, sudden death, all-cause mortality, risk of nonfatal and fatal MI, as well as secondary endpoints such as TG levels, systolic and diastolic blood pressure, and platelet aggregation [17].

Despite the myriad of evidence supporting EPA and DHA in CVD prevention, concerns about fish taste, smell, toxin content, radiation levels, allergies and eructation following fish meals are some factors that limit their therapeutic use [17]. Thus, attention has been given to the plant source of ω -3 PUFA, ALA. Dietary sources of ALA include canola, rapeseed, walnuts, perilla and leafy green vegetables; however, flaxseed (the focus of the current study) has the highest content of ALA relative to other dietary sources.

6. *Flaxseed*

6.1 *History of Flaxseed*

Flax or linseed (*Linum usitatissimum*) is an ancient crop that dates to 9000 – 8000 B.C.E. in Turkey, Iran, Jordan and Syria [226, 227]. However, evidence of its cultivation was first marked in Eastern Turkey around 7000 – 6000 B.C, where it was primarily used as a laxative or for bread and linen production [226, 227]. Currently, there are about 50 countries that grow flaxseed, and Canada is the largest producer, producing approximately a third of the 2 billion metric tons grown per year [227]. Current uses of flaxseed includes: varnish and paint-drying agent from flaxseed oil; animal feed and

fertilizer from flaxseed meal; and an ingredient in breakfast cereals and bread from the seed itself [226]. However, within the last 2 decades flaxseed has been increasingly utilized for its health benefits.

6.2 *Flaxseed components*

Flaxseed contains three functional components that have important health benefits: the ω -3 PUFA ALA, plant lignans such as secoisolariciresinol diglucoside (SDG), and non-starch polysaccharide such as soluble fibers or mucilage [228].

Flaxseed oil is the richest source of ALA, comprising approximately 51 – 55% of the oil content and 20% of the flaxseed's dry weight. Additionally, it has been reported in Canada that ALA content of flaxseed oil can peak at 61.9% [227]. Flaxseed's primary PUFA is ALA; however, it also contains small quantities of linolenic acid (LA), an ω -6 PUFA (15-18% of flaxseed oil). Flaxseed is considered a low-saturated fat food since its oil contains 73% PUFA, 18% MUFA, and 9% saturated fatty acids [228]. This oil content can fluctuate depending on environmental conditions (such as late frost, heat damage and drought), location and cultivar. Moreover, growing conditions also dictate the PUFA distribution of flaxseed.

With the exception of sesame seeds, flaxseed has the highest known lignan contents, which is nearly a hundred-fold higher than other dietary sources [229]. Regardless, flaxseed is the richest source of the lignan SDG and is approximately 1% of flaxseed's dry weight, varying between 0.6 and 1.8g/100g [230, 231]. It also contains minor amounts of other lignans, including pinoresinol, cinnamic acid glucoside, hydroxymethylglutaryic acid, lariciresinol and matairesinol, which collectively form the flax lignan complex (FLC) [231].

Finally, flaxseed contains both insoluble and soluble fiber components that make up approximately 6% of its dry weight [226]. Some of flaxseed's anti-atherosclerotic properties stems from its soluble fibers (mucilage/gum), which is about one quarter of the total dietary fiber available in flaxseed.

6.3 *Flaxseed components: structure and metabolism*

6.3.1 *Polyunsaturated Fatty acids*

Polyunsaturated fatty acids are naturally occurring long-chain fatty acids that contain >1 double bond within an aliphatic, hydrocarbon chain. The two biologically relevant families of PUFAs are the ω -3 and ω -6 PUFAs [232, 233]. Although they are similar in structure, the placement of the ω -3 PUFA's terminal double bond between 3rd and 4th carbon atoms from the methyl end confers differing biological properties to their ω -6 PUFA counterparts, which contain their terminal double bond between the 6th and 7th carbon atom [221, 222, 232]. Since mammals lack the enzymes (vegetable Δ 12 and Δ 15 desaturases) able to unsaturate the hydrocarbon chains at these position, both ω -3 and ω -6 PUFAs represent "essential fatty acids". The lack of these essential nutrients produces essential fatty acids deficiency syndromes, which results in dermatitis (desquamative rashes and hyperkeratotic dermatoses) [220, 221].

The simplest members of ω -6 and ω -3 families are LA (18:2 ω -6) and ALA (18:3 ω -3), respectively. After ingestion, absorption, transport into cells by specific fatty acid binding proteins and conversion by acyl-CoA synthases into thioesters, LA and ALA have three metabolic fates. Firstly, these PUFAs may be used for energy production through the classical β -oxidation pathways. Second, the produced fatty acid acyl-CoA thioesters may be used to synthesize neutral and polar lipids. Finally, LA and ALA can

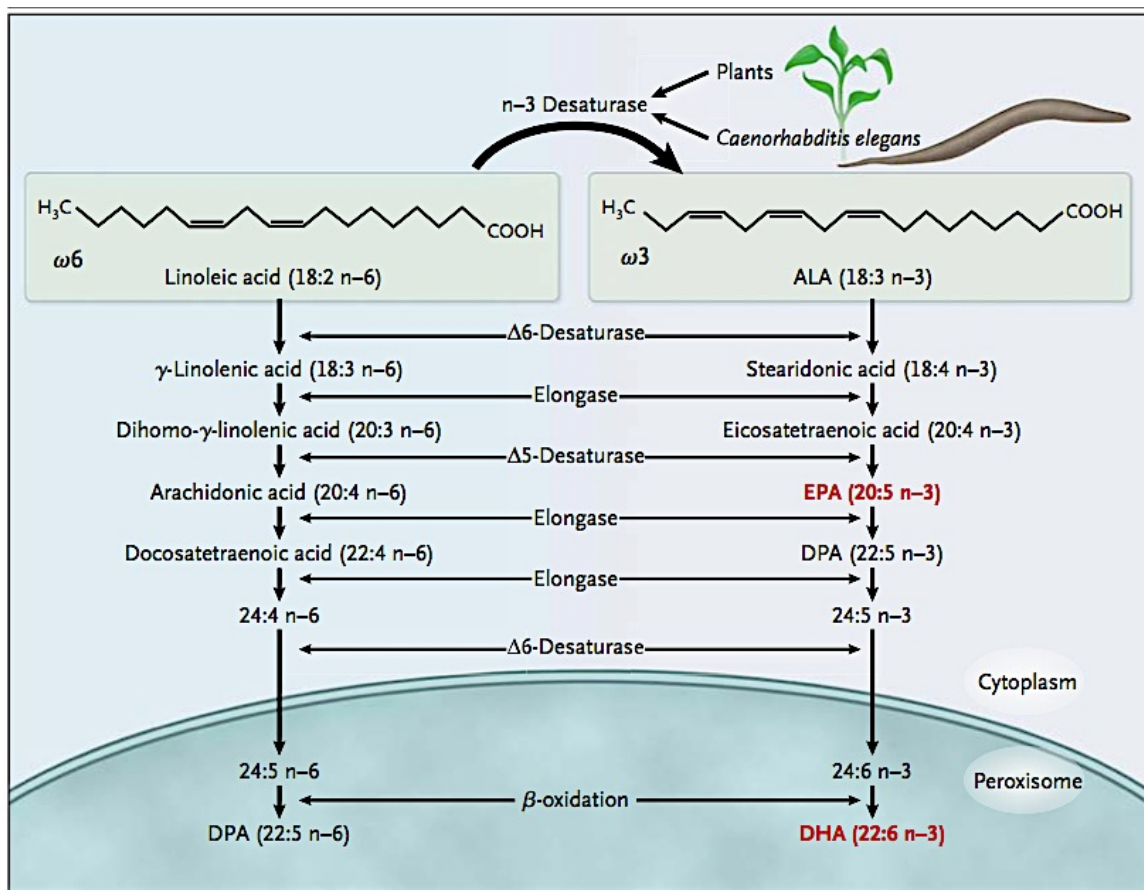


Figure 6. Elongation and desaturation of α -linolenic acid and linoleic acid. *Reproduced with permission from (De Caterina, R., n-3 fatty acids in cardiovascular disease. N Engl J Med, 2011. 364(25): p. 2439-50.), Copyright Massachusetts Medical Society [220].*

be used to generate long chain fatty acids (Figure 6).

With the exception of carnivores such as lions, most mammals can convert LA and ALA to biologically active long-chain derivatives [234, 235]. Conversion of these PUFAs mainly occur in the endoplasmic reticulum of many different tissues; however, conversion is most pronounced in the liver [221]. Both types of PUFAs compete for the same set of enzymes for elongation and desaturation. The first and rate-limiting step of ALA and LA metabolism is their conversion to stearidonic acid (18:4 ω -3) and α -linolenic acid (18:3 ω -6), respectively, by $\Delta 6$ -desaturase. After an additional two carbons are

added by an elongase, $\Delta 5$ -desaturase yields EPA (C20:5 ω -3) and arachidonic acid (C20:4 ω -6; AA). EPA can be further elongated to docosapentaenoic acid (C22:5 ω -3; DPA), which is subsequently used to produce DHA (C22:5 ω -3) [236]. Interestingly, DHA can be retro-converted back to EPA, and DHA seems to be a reserve of EPA since they produce similar cellular effects [179]. Although these PUFAs are acted upon by the same set of enzymes, ω -3 and ω -6 PUFAs cannot be interconverted by mammals. Thus, the amount of ALA and LA conversion depends on the bioavailability of these substrates, where an excess of ALA or LA will lead to increases in EPA, DPA and DHA or AA, respectively. Although ALA is the preferred substrate of $\Delta 6$ -desaturase, the conversion of ALA to stearidonic acid is kinetically slow. As a result, conversion of ALA into its longer-chain derivatives is inefficient in humans [179].

PUFAs may eventually be packaged into phospholipids destined to be embedded into biological membranes as polar lipids [221]. External stimuli, such as injury or infection, promote the activation of phospholipase A2 that releases both AA and EPA/DHA to serve as substrates for eicosanoid production (Figure 7) [232]. Eicosanoid production is mediated through the “orthodox” and the “unorthodox” pathways. The orthodox pathway involves the cyclooxygenase 2 (COX-2)-mediated and lipoxygenase-5 (5-LOX)-mediated production of eicosanoids, including prostaglandins (PG), thromboxanes (TX) and leukotrienes (LT) that exert a variety of biological effects. Arachidonic acid, EPA and DHA all compete as substrates for COX-2 and 5-LOX. However, unlike PUFA conversion, ω -3 fatty acids have a low binding affinity for these enzymes in comparison to AA. In endothelial cells AA is used by COX-2 to produce the 2-series prostanoids (PGI2 and TXA2), while in leukocytes and monocytes, 5-LO uses

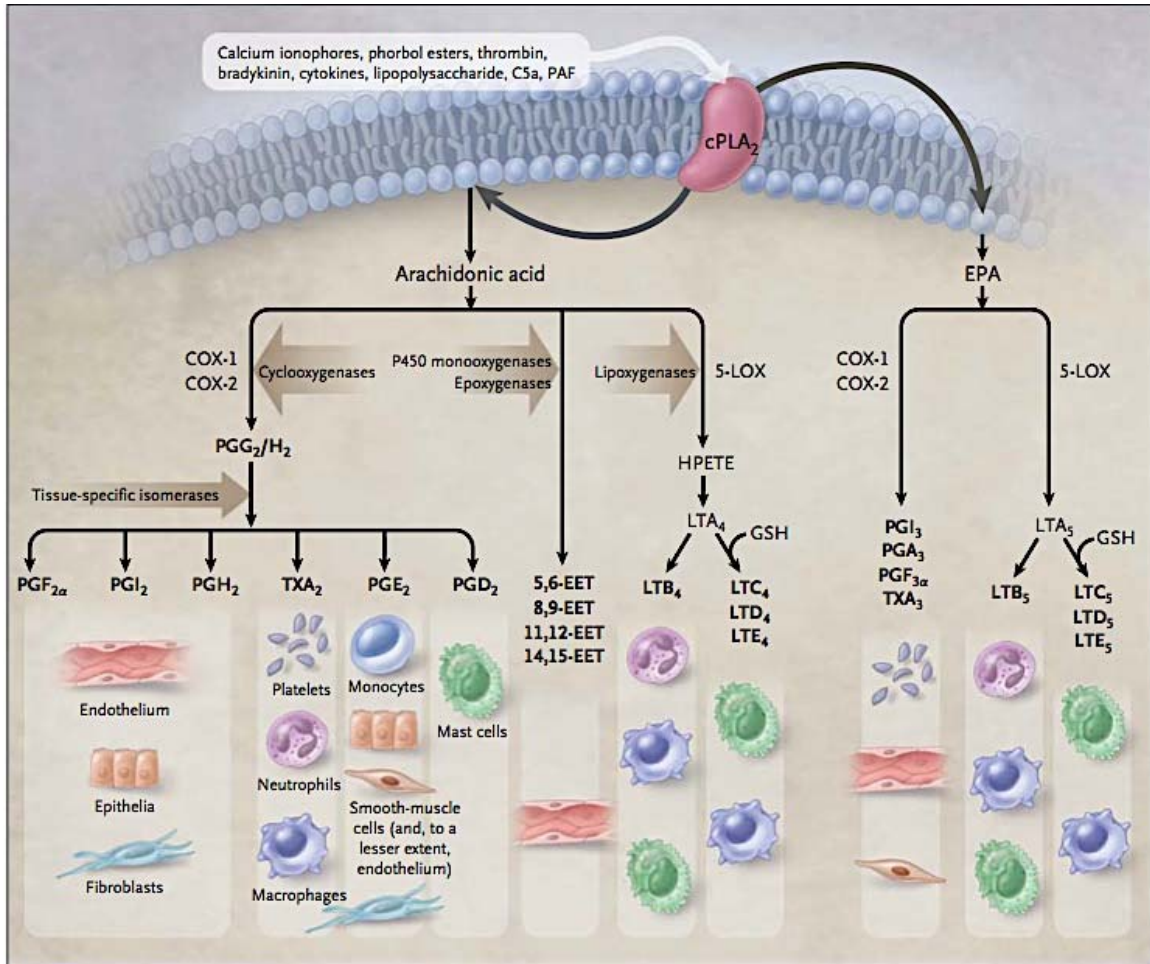


Figure 7. Eicosinoid production from arachidonic acid and eicosapentaenoic acid. *Reproduced with permission from (De Caterina, R., n-3 fatty acids in cardiovascular disease. N Engl J Med, 2011. 364(25): p. 2439-50.), Copyright Massachusetts Medical Society [220].*

AA to produce LTB₄ and sulfide-peptide LTs, such as LTC₄, LTD₄ and LTE₄. Alternatively, COX-2 within ECs use EPA as a precursor for the 3-series prostanoids (PGI₃ and TXA₂), whereas in leukocytes and monocytes, EPA is used by 5-LO for the synthesis of 5-series LTs, including LTB₅, LTC₅, LTD₅ and LTE₅ [221].

Although AA-derived eicosanoids are naturally occurring and needed to respond to various physiological and pathological stimuli, the overconsumption of foods rich in ω -6 fatty acids results in the overproduction of ω -6 PUFA-derived eicosanoids, inducing a

pro-inflammatory, pro-thrombotic and pro-aggregatory state that contributes to chronic diseases such as atherosclerosis [222, 232]. High consumption of ω -3 PUFA leads to the production of less potent eicosanoid equivalents. Thus, despite having the same biological affect, the production of ω -3 PUFA-derived eicosanoids reduces vascular tone, platelet aggregation and inflammation [221, 232].

The unorthodox pathway generates the production of “alternative eicosanoids” solely from ω -3 fatty acids, called resolution phase interacting products or resolvins (Figure 8). The combined activities of aspirin-acetylated COX-2 (or cytochrome P-450 monooxygenase) and 5-LO produces the E-series resolvins (RvE1 and RvE2) and 17R D-series resolvins (RvD1, RvD2, RvD3 and RvD4) from EPA and DHA, respectively. These eicosanoids resolve inflammation through the inhibition of apoptosis, superoxide generation, NF- κ B activation, platelet activation, integrin and immunoglobulin expression and the suppression of neutrophil transmigration and infiltration and vasoconstriction [221].

6.3.2 *Lignans*

Lignans are diphenolic compound that belong to the phytoestrogen family of naturally occurring plant sterols. Like most plant lignans, SDG is found as a diglucoside, being bound to sugars. SDG is also part of an ester-linked complex or oligomer containing 3-hydrozyl-3-methyl-glutaric acid and a number of cinnamic acid glycosides (usually ferulic or p-coumaric acid, and the flavonoid herbacetin) [229, 237]. Upon ingestion, the sugar moieties of SDG are hydrolyzed by bacterial or human β -glucosidase within the intestinal mucosal brush border to release aglycones such as secoisolariciresinol (SECO) (Figure 9). SECO and other aglyconse, like matairesinol, are

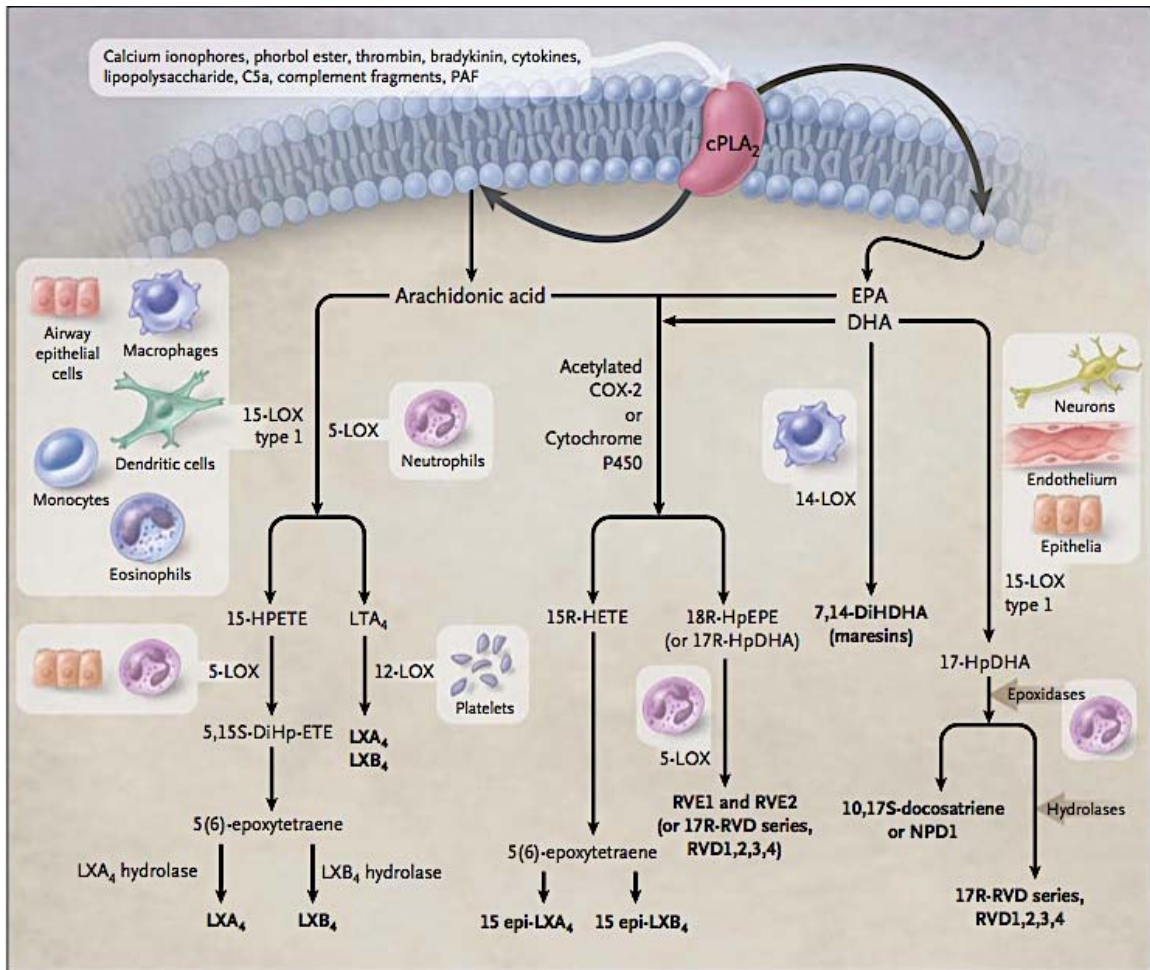


Figure 8. Production of the alternative eicosanoids, resolvins. *Reproduced with permission from (De Caterina, R., n-3 fatty acids in cardiovascular disease. N Engl J Med, 2011. 364(25): p. 2439-50.), Copyright Massachusetts Medical Society [220].*

further metabolized by bacteria and converted to mammalian lignan (enterolignans). SECO dehydroxylation produces enterodiol (ED), which can subsequently be oxidized to produce enterolactone (ENL). Matairesinol demetylation also yields ENL [229]. Both the efficiency of absorption and conversion of plant lignans greatly vary from person to person and are influenced by several factors. In humans, the efficiency of conversion within 24 hours ranges from 0 to 100 percent. In addition, intestinal pH, redox potential, transit time, genetic factors, and floral community will all contribute to inter-individual

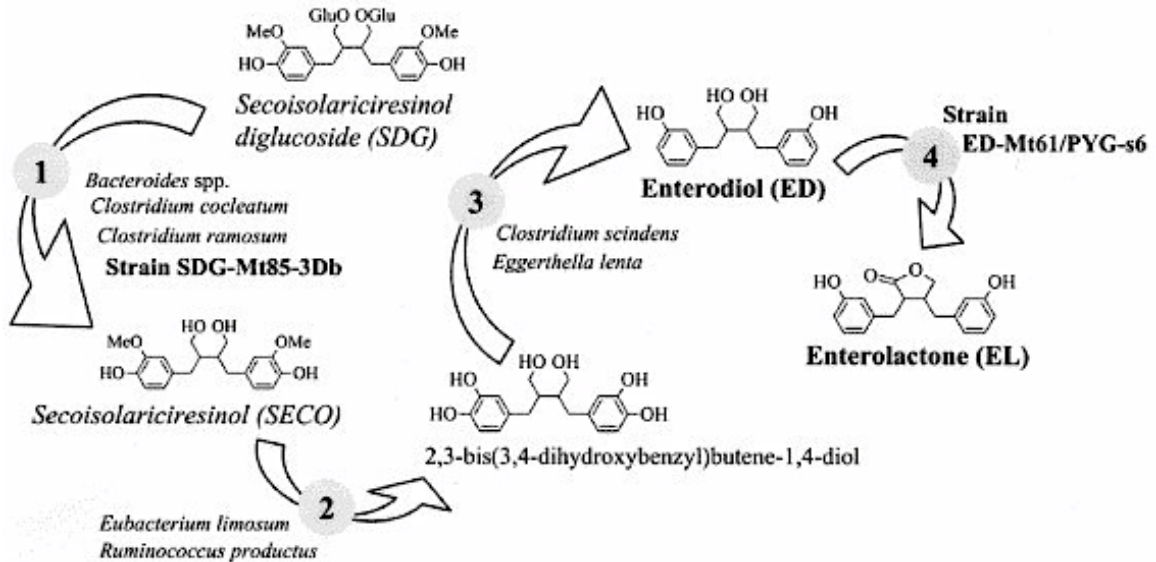


Figure 9. SDG intestinal conversion to the mammalian lignans enterodiol and enterolactone. © 1999. T Clavel, R Lippman, F Gavini, J Dore, and M Blaut. *Clostridium saccharogumia* sp. nov. and *Lactonifactor longoviformis* sp. nov., two novel human farcal bacteria involved in the conversion of the dietary phytoestrogen secoisolariciresinol diglucoside. *Systematic and Applied Microbiology*. Image used with permission from Elsevier on July 3, 2012 [238].

variation in lignan metabolism [229, 237]. However, the precise mechanisms responsible for the uptake of lignans in the small intestine remain unclear.

6.3.3 Dietary fiber

Dietary fiber is a type of complex carbohydrate or lignin, a large plant polymer built from *p*-coumaryl, coniferyl and sinapyl hydroxycinnamic alcohols, that resists hydrolysis and absorption during intestinal digestion [229, 239]. Dietary fiber exists in soluble and insoluble forms. Soluble fibers can be dispersed within water and are thought to influence blood lipoprotein levels and weight reduction. Insoluble fibers are thought to function as laxatives that aid in bowel regularity. However, it is becoming increasingly apparent that conclusively differentiating the physiological effects of soluble

and insoluble fibers is very difficult. Additionally, the mechanism of uptake and digestion are complex and remain unclear [239].

6.4 *Effects of flaxseed on cardiovascular disease*

The dietary intake of flaxseed has been associated with a decreased prevalence of CVD. Moreover, whole flaxseed or its functional components has been recommended in numerous health guidelines. For example, an ALA intake of 1.1 and 1.6 g/day has been recommended for women and men, respectively, by Health Canada (2003) and the US institutes of Medicine, Food, and Nutrition Board (2002) [227]. However, other researchers suggest adult ALA intake should be approximately 2.22 g/day¹. The American Health Association (AHA) recommends a daily fiber intake of 25-30 g². In addition, the Heart Foundation of Australia recommends the consumption of plant sterol enriched foods for patients who are at high risk of CVD [240]. Likewise, the National Cholesterol Education Program and the AHA has recognized the beneficial effects of dietary phytosterols intake in hypercholesterolemic adults [228].

In animal studies whole flaxseed supplementation have shown anti-atherosclerotic properties with or without appreciable augmentation of lipid profiles. Prasad [241] showed that flaxseed consumption (7.5 g/kg daily) significantly decreased the development of diet-induced atherosclerotic plaques in the aorta of rabbits by 46% and reduced polymorphonuclear leucocytes, an indicator of reactive oxygen species load,

¹ To obtain 2.22g/day of ALA from flaxseed intake would require the consumption of approximately 11.1 g of flaxseed per day. This amount of flaxseed consumption can be easily achieved and incorporated into daily routines.

² To obtain 25-30 g/day of fiber from flaxseed intake would require the consumption of 415-500 g of flaxseed per day. This amount of flaxseed is unlikely be tolerated by any individual. Daily fiber intake of 25-30 grams can be achieved from the ingestion of other dietary fiber sources other than flaxseed.

without significant reductions in serum cholesterol levels. Likewise, Dupasquier et al. [6] revealed that a 10% flaxseed-supplemented diet (12.5 g/day) can suppress cholesterol-induced atherogenesis as well as protected vascular contractile function and vasorelaxation within rabbits. These changes occurs independent of changes in lipid profile. Additionally, flaxseed (0.4 g/day) effectively inhibited the expression of inflammatory markers such as Il-6, Mac-3, VCAM-1 and the proliferating cell nuclear antigen (PCNA) in aortic lesions from LDL receptor-deficient mice [15]. However, in mice, reductions in atherosclerosis and concurrent decreases in aortic inflammation was accompanied with significant ($P>0.05$) decreases in TC. Likewise, consumption of flaxseed in ovariectomized Golden Syrian hamsters reduced serum cholesterol levels and reduced atherosclerosis in a dose-dependent manner [242].

A number of clinical trials have shown that whole flaxseed intake can effectively decrease TC and LDL. Studies evaluating short-term (1 to 3 months) flaxseed consumption in hyperlipidemic individuals have shown that medium to high (15 grams/day to 50 grams/day) flaxseed supplementation can significantly reduce the serum levels of LDL (3.9% - 18%), triglyceride (TG; up to 36.3%), and total cholesterol (6% - 17.2%) [243-246].

Similar findings have been produced in both post-menopausal and menopausal women. A recent double-blind, randomized, controlled trial of men and postmenopausal women provided a flaxseed diet (40 g/day) for 10 weeks showed that flaxseed supplementation reduced LDL-C (7%) without augmenting inflammatory markers, including IL-6 and high-sensitivity C-reactive protein (hsCRP) [247]. Another randomized, double-blind, placebo-controlled trial in healthy menopausal women showed

that 12 months of supplementation with flaxseed (40 g/day) increase apoAI (4.4%) and decreased LDL peak particle size [248]. Finally, in a hypercholesterolemic Native American postmenopausal population flaxseed consumption (30 g/day) exhibited a 7% and 10% reduction of TC and LDL, respectively, without alterations in HDL and TG levels [249].

Studies evaluating the effects of flaxseed on secondary endpoints such as hypertension have shown that it may have potential efficacy in reducing mean arterial blood pressure. Pachos and colleagues reported that 12 weeks of dietary flaxseed supplementation (8 g/day of ALA) resulted in a significant decrease in systolic and diastolic blood pressure in dyslipidemic patients [231].

Despite the myriad of studies showing that whole flaxseed supplementation has the potential to reduced cardiovascular disease risk factors, more studies are needed to deduce its effects on primary ends points such as non fatal and fatal MI as well as cardiac mortality and morbidity. In addition, the effects of whole flaxseed (or even flaxseed components) on surrogate endpoints such atherosclerotic plaque regression has not been fully assessed by clinical or animal studies. Thus, there is a need of translatable animal studies to evaluate the efficacy of flaxseed as a potential nutritional intervention for the regression of atherosclerotic plaques.

7. *Animal models to study regression*

In 1908, Ignatowski was the first investigator to experimentally induce atherosclerosis in an animal model. In this study, atherosclerosis was mildly induced by feeding rabbits meat, eggs, and milk. Afterwards, Lubarsh and Steimbiss induced

atherosclerosis by feeding rabbits a diet including liver, adrenal gland and muscle. From these sets of experiments many researchers believed that animal proteins initiated plaque formation [250]. However, Clark and Newburg solidified that cholesterol rather than proteins was the culprit in plaque development by inducing moderate atherosclerosis in rabbits after feeding a cholesterol-supplemented diet (507 mg/day; administered in capsules) for up to 87 days. Furthermore, Meeker and Kesten observed human like atherosclerotic lesion formation after feeding rabbits a diet with vegetable oil fortified with 60 or 250 mg of cholesterol [251].

Since these pioneering studies, a variety of animals including mice, rats, rabbits, turkeys, pigeons, chickens, swine, dogs and nonhuman primates such as chimpanzees, baboons, rhesus monkeys, cynomolgus macaque, stump-tail macaque, squirrel monkeys, and cubus monkey have been utilized to study atherogenesis [252]. Although genetically altered mouse models such as the LDL receptor-knockout and apoE-knockout mice are attractive tools for studying the effects of interventions on atherosclerosis, these models are deficient in key machinery involved in plaque regression. Thus, rabbits have been commonly utilized to study the effects of nutritional interventions on atherosclerotic plaque regression.

Low initial cost, low maintenance cost, ease of handling, short duration to reproductive maturity, a well-characterized genetic background, and shared pathophysiology of human diseases are all essential criteria for an ideal animal model for human atherosclerosis [253]. Humans and rabbits share a variety of similarities in lipoprotein metabolism including: apoB100-containing VLDL production by the liver, apoB composition and plasma CETP activity [253]. In addition, the high dietary

cholesterol absorption rates and rapid development of aortic lesions and low maintenance cost makes the cholesterol-fed rabbit model a formidable model for studying atherosclerosis [254]. Earlier studies tended to use 1 or 2% cholesterol to induce plaque development. However, it is now understood that atherosclerotic lesion formation in rabbits is not dependent on the level of administered cholesterol but rather on the cumulative exposure to hypercholesterolemia. Currently, lower concentrations of cholesterol in animal feed (0.3 to 0.5 %) are used [255, 256]. Generally, rabbit lesions in young animals (approximately 4 months) resemble fatty streaks with a large accumulation of sterol filled foam cells. However, advanced lesion development with complicated, mature features such as fibrosis can develop in aged rabbits (3-5 years old) [257]. Other additional complications like plaque hemorrhage, ulceration, calcification and thrombus formation do not naturally occur in rabbits. However, these features can manifest with additional manipulations such as vessel balloon injury, epinephrine and vitamin D administration, and alternative feeding of cholesterol-containing and normal diets [258-260].

Despite the similarities between rabbits and human, rabbits have many physiological differences with humans and are not completely “ideal”, like all experimental models. Humans tolerate cholesterol intake better than rabbits, which do not usually eat large amounts of cholesterol. Upon cholesterol feeding rabbits produce marked hypercholesterolemia with plasma cholesterol levels exceeding 1,000 mg/dl, a value that would be hard to attain even in patients with familial hypercholesterolemia [252, 254, 255]. Additionally, atherosclerosis typically progresses over a long period of time in humans, while lesions in rabbits are produced within a matter of weeks.

Moreover, the distribution of lesions within the aorta differs between rabbits and humans. Rabbits generally have greater amounts of atherosclerosis in the abdominal aorta as opposed to the greater amounts in the thoracic aorta seen in humans [254]. Finally, rabbits are apoAII and hepatic lipase-deficient [250].

Nevertheless, rabbits represent an efficacious animal model to study the effects of nutritional interventions on atherosclerotic plaque regression because they do not require genetic manipulation to create plaques as with rodent models and have been preferentially utilized by several investigators evaluating nutrition and atherosclerosis development [253]. Although many studies have utilized nutritional interventions to induce plaque regression [18-22], all have prematurely initiated their interventions before there is clear evidence of plaque growth plateau. If a rabbit is supplemented with dietary cholesterol followed with cholesterol withdrawn from the diet, atherosclerotic lesions actively continue to progress for many weeks. These lesions only begin to stabilize weeks after the removal of cholesterol from the diet and as the hypercholesterolemia resolves [23-27]. Thus, if an intervention is imposed immediately after the withdrawal of cholesterol from the diet as has been done in previous studies of regression [18-21, 28], any effects observed will reflect an inhibition of plaque progression and not an effect on plaque regression.

Hypothesis

A flaxseed-supplemented diet will:

1. Accelerate the regression of diet-induced atherosclerotic plaque of rabbits
2. Decrease the serum markers that are associated with inflammation
3. Improve the pathological complications induced by cholesterol feeding such as vascular function abnormalities

Objectives

The objective of the present study was to:

1. Identify a suitable dietary regimen in rabbits that show clear evidence of plaque growth stabilization
2. Determine the ability of dietary flaxseed to induce or accelerate atherosclerotic plaque regression
3. Discern whether flaxseed supplementation can reverse any cholesterol-induced vascular contractile abnormalities
4. Determine whether dietary flaxseed supplementation can ameliorate the detrimental physiological impact that cholesterol feeding has on platelet aggregation and inflammation

Methods and Materials

1 Determination of plaque development plateau in rabbits

1.1 Animals and dietary intervention

Male New Zealand white (NZW) rabbits (Spilak farms, Manitoba, Canada), weighing between 2.7 to 3 kg, were randomly assigned to 1 of 8 dietary regimens (Figure 10)³. All animals were maintained on a 1% cholesterol-supplemented diet (Ren's Pet Depot, Guelph, ON Canada) for 4 weeks. A subset of animals was immediately sacrificed. The remaining animals were provided with regular rabbit chow (Nutrena, nature wise performance rabbit formula, Manitoba, Canada) and sacrificed at intervals up to 28 weeks after removal from cholesterol feeding. Serum lipid and *en face* analyses (described in Methods and Materials: Section 1.2 and 1.4, respectively) were performed after sacrifice. Upon arrival, rabbits were housed in individual cages in rooms with controlled temperatures, humidity, and a 12-hour light/ 12-hour dark cycle. Rabbits were fed 125 g of their respective dietary regimens per day. Water was supplied *ad libitum*.

Experiments were reviewed and approved by the University of Manitoba Protocol Management Review Committee, in the accordance with the Canadian Council on Animal Care Guidelines.

³ The sample size of each group is not consistent. In a pilot study, animals were maintained on eight weeks of regular diet to deduce when atherosclerotic plaque regression ceased. Only a few animals (2-4) were utilized to gain insight into plaque dynamics after long-term cessation from dietary cholesterol feeding. Additional animals were required to increase the sample sizes and statistical power. Moreover, it was essential to determine that plaque regression did not occur sooner than eight weeks. Thus, six additional animals were used to satisfy the former dilemma and answer the latter inquiry.

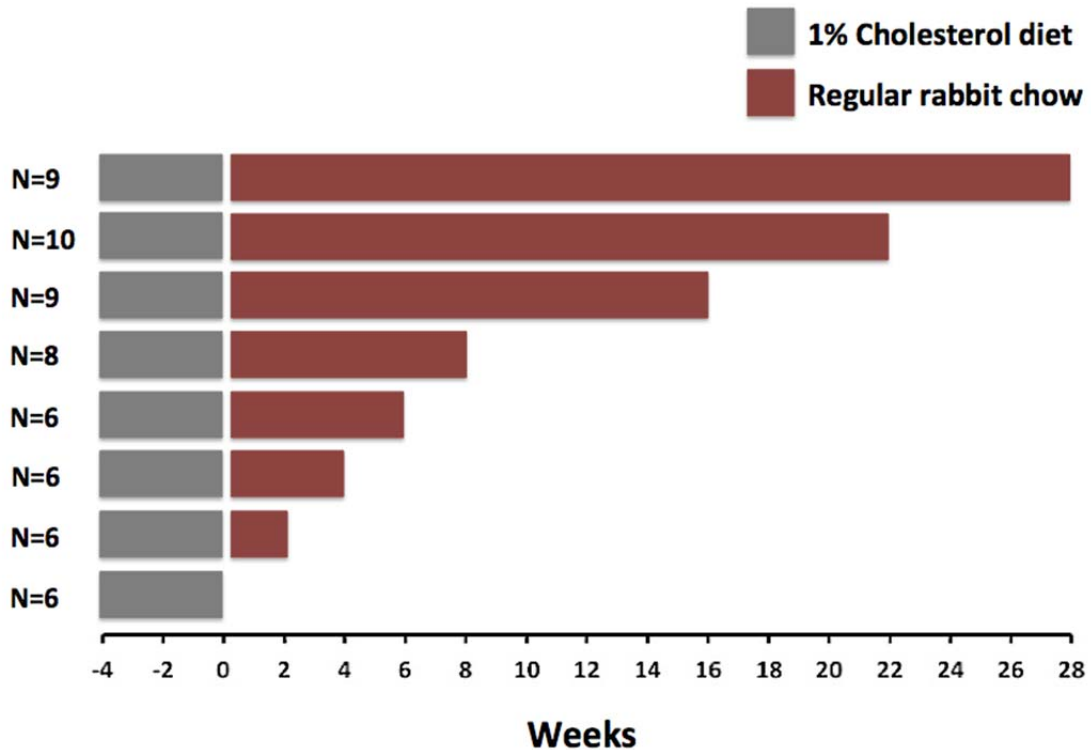


Figure 10. Feeding regimens used to evaluate the effects of long-term cholesterol withdrawal on atherosclerosis after 4 weeks of a high cholesterol diet.

1.2 Blood sampling and analysis

Blood samples were obtained at the time of sacrifice from the jugular vein while the animals were anesthetized with isofurane gas (5% with oxygen/min). The samples were collected in vacutainer tubes containing 7.2 mg of K₂EDTA (Becton Dickinson, Franklin Lakes, New Jersey, USA). Blood samples in EDTA vacutainer vials were immediately inverted, placed on ice and afterwards centrifuged at 1800 rcf (3160 rpm) for 15 minutes at 4°C. Aliquots of serum were placed into cryogenic vials, flash frozen in liquid N₂, and stored at -80°C. The serum was used to determine total cholesterol, triglyceride and CRP levels. Serum cholesterol and triglycerides levels were measured enzymatically using commercial kits (Genzyme diagnostics, Prince Edward Island, Canada).

The circulating levels of CRP were measured by Western immunoblotting analysis. Serum from rabbits was diluted five-fold with RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.05% sodium deoxycholate, 1 mM benzamide and a protease inhibitor cocktail). A two-fold dilution was performed with 2× Sample buffer and enough volume was prepared to analyze 4 µl of plasma by SDS-PAGE electrophoresis. Proteins were immediately transferred onto a BioTrace™ nitrocellulose membrane (Pall Life Sciences, Ann Arbor, MI, USA) using a semidry transfer protocol. All membranes were blocked with 10% skimmed milk in TBS-T (50 mM Tris base, 200 mM NaCl, 0.5% Tween-20) overnight at 4°C. Blots were probed overnight at 4°C with a chicken anti-CRP primary antibody (Abcam Inc. Cambridge, MA USA: ab46819) that was diluted to 1:1000 in 2% partially skimmed milk in TBS-T. After three fifteen minute washes with TBS-T, the membranes were incubated for one hour with a horseradish peroxidase (HRP) conjugated goat-anti-chicken secondary antibody (Bethyl Laboratories Inc. Montgomery, TX USA: A30-106P). After an additional 3× fifteen to twenty minute washes, blots were incubated with the HRP substrate Luminata™ forte (Millipore Corporation, Billerica, MA, USA) for 5 minutes and the signal was quantified by densitometric analysis using Quantity One software (Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario, Canada) on a Bio-Rad GS-670 imaging densitometer. CRP levels were represented as percentages of serum obtained from a rabbit fed a 1% cholesterol diet for 4 weeks. The efficiency of transfer was verified using the Ponceau S staining solution (Sigma-Aldrich, USA).

1.3 *Tissue removal and preparation*

Prior to anesthetization, rabbits' neck, chest and abdomen were shaved in preparation for tissue removal. After blood sample collection, aortas were removed from the proximal aspect of the aortic arch to the base of the diaphragm. The aortas were immediately placed in Krebs-Henseleit solution containing (115 mM NaCl, 25 mM NaHCO₃, 1.38 mM KH₂PO₄, 2.5 mM KCl, 2.46 mM MgSO₄, 1.9 mM CaCl₂, 11.2 mM dextrose, pH 7.4), cleaned of gross adventitial tissue, and prepared for the *en face* analysis.

1.4 *Evaluation of Aortic lesion area*

The extent of plaque accumulation on the vessel surface was determined by the *en face* analysis. The aorta was longitudinally opened, fastened in place with knitting needles on a sterile tissue pinning platform, and digitally photographed with a Nikon Coolpix 995 camera (Nikon, Tokyo, Japan). The camera settings were as follows: fine focus with both ISO and shutter speed set to automatic (zoom was used when necessary). Once a digital image was made, the image was converted to a gray-scale image and the photo's levels, which move and stretch the brightness levels of an image histogram, were adjusted using Adobe Photoshop CS3 (Adobe Systems Inc., San Jose, CA, USA). Luminal images were subsequently analyzed using the Nikon imaging software (Nikon Instruments Inc., Melville, NY, USA). The percent lesion area was tabulated from the fraction of area covered with lesions relative to the total area of the aorta.

2. *Effects of dietary flaxseed intake on cardiovascular risk and atherosclerotic plaque regression*

2.1. *Flaxseed supplemented diet preparation*

Diets were prepared by mixing an appropriate amount of flaxseed to regular rabbit chow to produce a 10% (wt/wt) flaxseed-supplement diet. The flaxseed was provided by Pizzly's Milling, Augusville, Manitoba, Canada and contained approximately 53% ALA. Firstly, a coffee grinder was used to mill the flaxseed prior to its mixture into regular rabbit chow. The regular diet was moistened with water and the required amount of

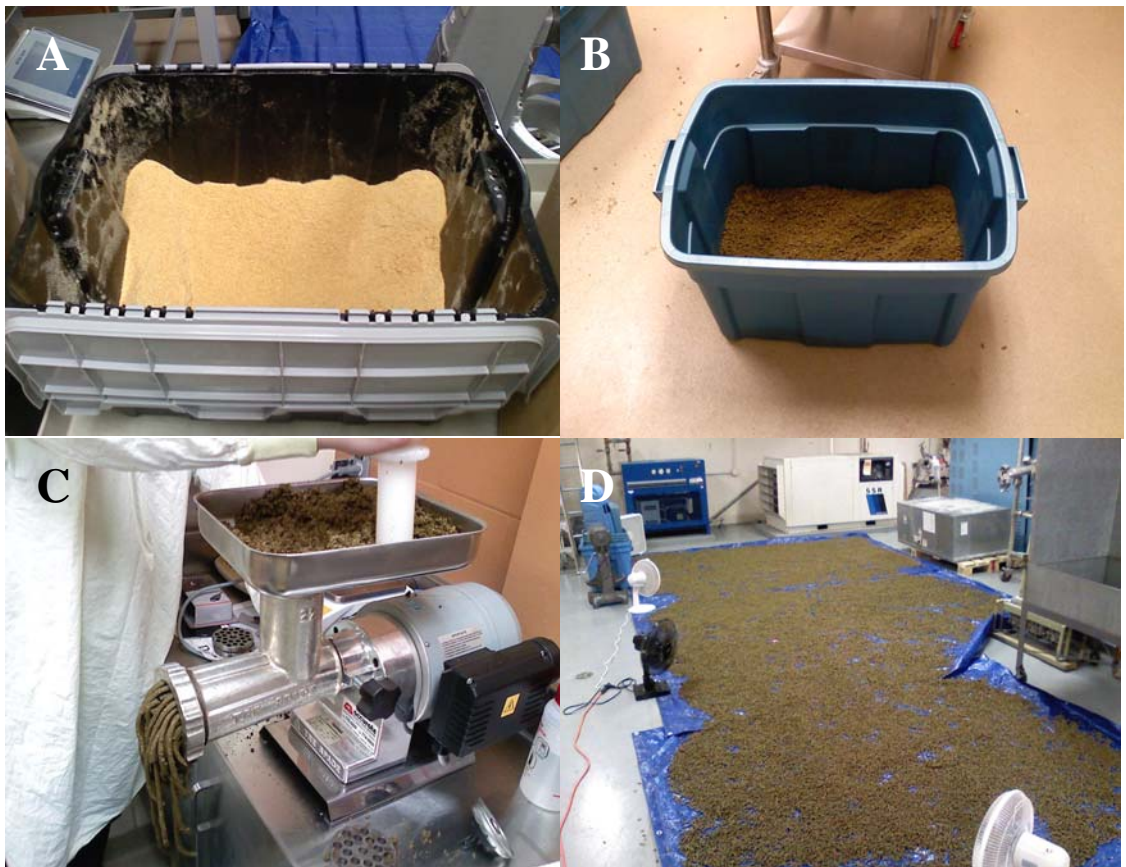


Figure 11. Preparation of flaxseed supplemented diet. Flax was ground (A) and mixed with standard rabbit chow (B). The mixture was repelleted with a commercial meat grinder (C) and fan-dried (D).

ground flaxseed was hand-mixed and later manually blended with a meat mixer. The mixed components were repelleted with a commercial grade electrical meat grinder (Cabela’s Canada, Winnipeg, MB, Canada), laid on tarps and fan dried (Figure 11). Once dried the diets were collected into storage bins and refrigerated at 4°C. The percentage of flaxseed incorporated into standard animal chow (10%) reflects the amount used in other animal studies that shows cardiovascular and therapeutic benefits [243, 261].

2.2 *Animals and dietary intervention*

Sixty albino male NZW rabbits were randomly assigned to one of six experimental groups (Figure 12). Control rabbits (Group I) were maintained on regular

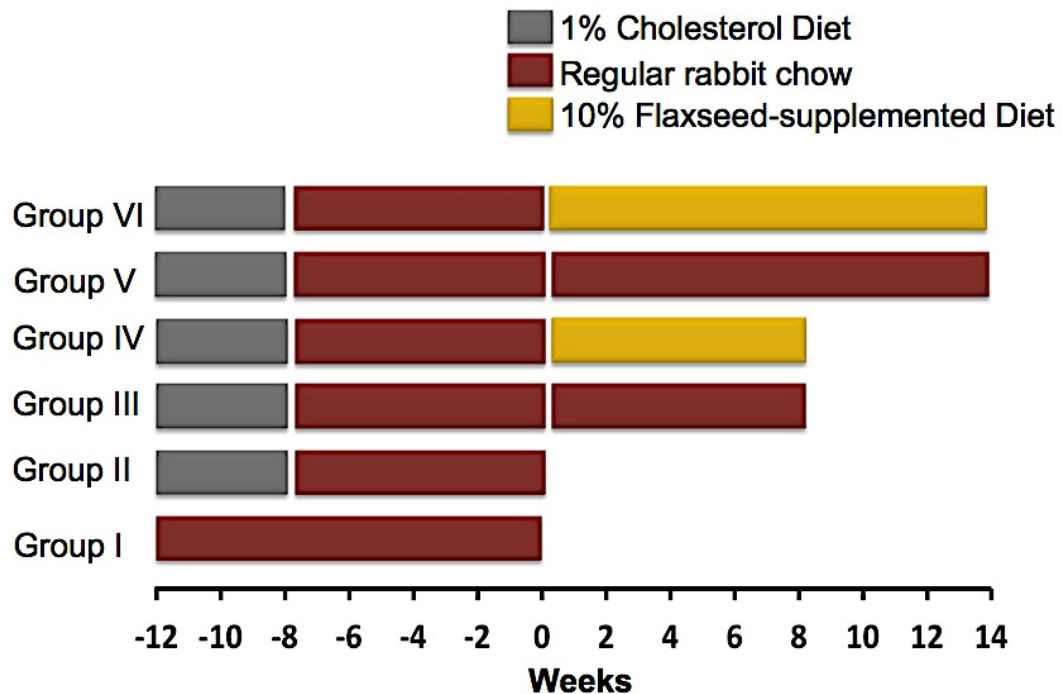


Figure 12. Dietary regimens utilized to determine the effects of flaxseed on atherosclerotic plaque regression.

rabbit chow for 12 weeks. All other animals received four weeks of a 1% cholesterol-supplemented diet for the initiation of plaque formation followed by 8 weeks of regular rabbit chow to steady plaque progression. A subset of animals was immediately used at this time point of plaque stabilization (Group II). The remaining animals were given an additional 8 or 14 weeks of regular rabbit chow (Group III and V, respectively) or animals were given 8 or 14 weeks of a 10% flaxseed-supplemented diet (Group IV and VI, respectively). Upon arrival, rabbits were subjected to the previously mentioned conditions. Rabbits were fed 125 g of their respective dietary regimens per day. The amount of milled flaxseed in the diet provides a dose of approximately 2.72 g ALA per day, an amount based data from a previous study [262]. Water was supplied *ad libitum*.

Again experiments were reviewed and approved by the University of Manitoba Protocol Management Review Committee, in the accordance with the Canadian Council on Animal Care Guidelines.

2.3 *Blood sampling and analysis*

Blood samples were obtained as described above (Methods and Materials: Section 1.2). The samples were collected in vacutainer tubes containing either 7.2 mg of K₂EDTA or 145 µl of 0.150 M citrate (Becton Dickinson, Franklin Lakes, New Jersey, USA). Blood samples in EDTA were treated as previously described (Methods and Materials: Section 1.2). Serum from EDTA vials was used for total cholesterol, triglyceride, CRP and fatty acid analyses. Plasma from citrate vials were used to evaluate the diet induced changes in platelet aggregation.

Serum cholesterol and triglycerides levels were measured enzymatically using commercial kits (Genzyme diagnostics, Prince Edward Island, Canada). Expression of CRP were measured as described above (Methods and Materials: Section 1.2).

Fatty acids were extracted from serum samples and derivatized according to the Lepage and Roy method [263]. Within a glass test tube 100 μ l of plasma was added to 2ml of methanol-toluene (4:1). The solution was vortexed and 200 μ l of acetyl chloride was subsequently added in a drop wise fashion. All samples were re-vortexed, sealed and heated to 100°C for one hour, during which tubes were vortexed at 15 minute intervals. After an hour of cooling, the solution was neutralized with 5 ml of 6% K₂CO₂. Samples were remixed and centrifuged for 5 minutes at 5000 rpm at 22°C to clearly separate the aqueous and organic layers. The upper toluene layer containing the resultant fatty acid methyl esters was collected into an autosampler vial. Fatty acid methyl esters were then used for gas chromatography (GC) on a Varian CP-3800 GC w/ flame ionization detector and CP-Sil 88 capillary column 60m x 0.25 mm x 0.20 μ m. 1 μ l of sample was injected at a flow rate of 1.5 ml/min. Split ratios were 1:5 initially, 1:50 after 0.01 minutes, and 1:5 after 1 minute. Pressure varied to maintain flow rate over the temperature program. The oven temperature was maintained at 111°C for 1 min, increased by 20°C/min to 170°C, raised again by 5°C/min to 190°C and finally increased by 3°C/min to 225°C. It was maintained at that temperature for 10 min, for a total run time of 29.62 minutes. Equipment was standardized using GLC 462 (Nu-Chek Prep, Inc.). The fatty acid content of the sample was identified comparison with authentic standards (NuCheck Prep, Elysian, MN, USA). The internal standard used was C19:0 (Nu-Check Prep, Inc.).

Blood samples in citrate vacutainer vials were immediately inverted, incubated for 30 minutes at room temperature (25°C), and centrifuged at 100 rcf for 15 minutes. The separated platelet-rich plasma fraction was obtained. Further centrifugation of the sample at 2400 rcf for 15 minutes separated the platelet-poor plasma. Equal volumes of the platelet rich and platelet poor plasma fractions were aliquoted into separate cuvettes. Either ADP or collagen was added to the platelet rich plasma such that the final concentrations were 15 μ M and 7.5 μ M or 4 μ g/ml and 8 μ g/ml, respectively. Upon addition of ADP or collagen, samples were immediately measured in a Chrono-log aggregometer. The platelet-poor plasma served as the blank and was treated in an identical fashion. Both percentage of maximal aggregation and rate of aggregation were obtained by using the Aggro/Link (v. 4.75) software.

2.4 *Tissue removal*

After blood sample collection, aortas were immediately removed, placed in Krebs-Henseleit solution, cleaned of gross adventitial tissue, and prepared for vascular function, *en face*, cross-sectional, total cholesterol, and fatty acid content analyses.

2.5. *Experimental assessment of Vascular Function*

The TSZ-04 Isolated Tissue Bath System (Experimental Ltd. Budapest, Hungary) was used to assess vascular function of aortic rings. Two 3 mm rings from immediately below the aortic arch were taken for vascular function analysis. The FSG-01 force/displacement transducer (Experimental Ltd. Budapest, Hungary) was set to a sampling rate of 100Hz -10ms and calibrated with 5, 10 and 20 grams, calibrating the unit between 0-5V/g. Changes in tension was resolved with the Experimentria EXP-D isolated organ amplifier (Experimental Ltd. Budapest, Hungary) and the Isosystem 1.0

chart as part of the Solution Pack for Experimental Ltd. (S. P. E. L) recording and analyzing software system (Experimental Ltd. Budapest, Hungary). Aortic rings were subsequently fastened to the organ bath and force transducer, equilibrated 3 times at 6.0 grams of tension for 15 minutes with Krebs-Henseliet solution (37°C), and aerated with 95% O₂ and 5% CO₂.

Following equilibration, aortic rings were brought to a basal tension of 6.0 g and contracted three times with the 46 mM KCl solution (78.7 mM NaCl, 22 mM NaHCO₃, 1.38mM NaH₂PO₄, 46 mM KCl, 4.1 mM MgSO₄, 2.51 mM CaCl₂, and 5.61 mM dextrose, pH 7.4). Each contraction was followed by a washout period, when rings were perfused with fresh Krebs-Henseliet solution (37°C), for the return of ring tension to basal levels. Increasing concentrations of norepinephrine (10⁻⁹ to 10⁻⁴ M) were used to construct a dose response curve. After an additional washout period, aortic rings were contracted with 10⁻⁶ M NE.

The ability of the tissue to relax was then tested by the additional administration of 10⁻⁸ to 10⁻⁵ M Ach, an endothelial-dependent mode of relaxation. Following an additional washout and pre-contraction with 10⁻⁶ M NE, equivalent concentrations of sodium nitroprusside (SNP) was used to test the vessels responsiveness to endothelial-independent relaxation. After the end of each experiment aortic rings were crudely dried with a kimwhipe to remove excess fluid and the tissue's wet weight was calculated. The extent of vasoconstriction and vasodilation was ascertained with the force transducer and recorded as mechanograms of tension [tension (g)/ tissue wet weight (g)].

2.6 *Evaluation of atherosclerotic lesion area*

The extent of plaque accumulation on the vessel surface was determined by the *en face* analysis, which is described above (Methods and Materials: Section 1.4). The only difference was the aorta was crudely reconstructed with the rings removed for assessing vascular response.

2.7 *Tissue sectioning and staining*

Following the *en face* analysis the opened aortic rings and aorta were prepared for sectioning. Three rings, one from each proximal, medial and distal areas of the aorta, were immersed in 4% paraformaldehyde (PFA) (Alfa Aesar, Ward Hill, MA, USA) solution. Likewise, an additional three rings were placed in a formalin + 10% neutral buffered solution (Sigma diagnostics, St. Louis, MO, USA) and stored at 4°C for 24 to 48 hours. The PFA or formalin was removed and the tissue was washed three times with 1× phosphate buffered saline (PBS) solution (Fisher Scientific BioReagents, FairLawn NJ, USA). The tissue was afterwards immersed in a 30% sucrose solution and stored at 4°C for 24 to 48 hours. The rings were removed for the sucrose solution and immediately submerged in Tissue-Teck Optimal Cutting Temperature (OCT) compound (Sakura Finetek USA Inc., Torrance, CA, USA) and subsequently frozen at -80°C. The rings were sectioned on a Shandon Cryotome and mounted on Fisherbrand® Superfrost®/Plus precleaned microscope slides (Fisher Scientific, Pittsburgh, PA, USA).

To demonstrate intimal lipid accumulation, sections were stained with Oil Red O (Sigma-Aldrich Inc., St. Louis, MO, USA). The OCT compound was rinsed off of microscope slides with tap water. These slides were placed in a staining rack containing 85% polypropylene for 5 minutes, stained in Oil Red O dissolved in polypropylene for 3

minutes, and afterwards rinsed in 85% polypropylene. Afterwards, the slides were rinsed with double-distilled (dd) H₂O and stained with hemotoxylin (Fluka Biochemika, Steinheim, Switzerland) for 10 minutes. Finally, slides were rinsed with tap water and ddH₂O and prepared for microscopy. Digital images were taken with a Nikon Coolpix 995 camera and a tabletop dissecting microscope. The camera settings were as follows: fine focus with both ISO and shutter speed set to automatic (zoom was used when necessary). The image was analyzed using Nikon imaging software (Nikon Instruments Inc., Melville, NY, USA). The percent Oil Red O staining was tabulated by measuring the area stained with Oil Red O and dividing that value by the total area of the cross-section.

2.8 *Aortic lipid extraction and quantification*

Lipids from whole aortas were extracted according to an adapted Folch method [264]. The tissue was thawed to 25°C and minced at 0°C for two minutes. The minced tissue was then homogenized 3 times in 5 mls of chloroform-methanol (2:1) at 4°C, which was collected and transferred to a separatory funnel. An additional 5 ml was used to rinse the homogenate and was subsequently added to the separatory funnel. 4.2 ml of 0.73% NaCl was added to the separatory funnel and its contents were vigorously mixed. The resulting emulsion was allowed to separate for 12 hours or overnight. The organic phase was collected, dried with sodium sulfate, and filtered. The remaining solvent was removed using rotary evaporation. Lipids were re-solubilized in 1ml of chloroform, transferred to a storage vial, and stored at -80°C. The amount of lipids per gram of tissue was calculated. From this lipid extract both total aortic cholesterol content and aortic fatty acid composition were ascertained.

Aortic cholesterol content was measured enzymatically using commercial kits (Genzyme Diagnostics, Prince Edward Island, Canada). A 100 μL aliquot of the lipid extract was dried under N_2 (g) and re-suspended in 200 μL of 0.9% NaCl containing 0.5% triton-X. The samples were sonicated for 30-40 minutes to create a cloudy emulsion. An aliquot of the emulsion was diluted (if necessary) and used to quantify the aortic cholesterol content.

Fatty acid methyl esters were obtained and resolved as described above (Methods and Materials: Section 2.3).

3. *Statistical analysis*

All data (from Methods and Materials: Section 1 and 2) are expressed as mean \pm standard error (SE). Statistical comparisons were made using a one-way analysis of variance, followed by the Student-Newman-Kuels *post hoc* test with $P < 0.05$ considered statistically significant.

Results

1. Determination of plaque dynamics after cholesterol withdrawal

1.1 Weight Gain

All animals consumed 125 g of diets allotted for their daily intake. Weight gain between all animals remained consistent during cholesterol feeding and after cholesterol withdrawal and there were no significant differences observed amongst any of the groups (Figure 13).

1.2 Serum lipid profiles

After four weeks of cholesterol feeding, serum cholesterol levels markedly increased (Figure 14A). Maintenance on regular rabbit chow after cholesterol feeding significantly decreased cholesterol levels in a stepwise fashion until baseline levels were achieved after 8 weeks of withdrawal. Similarly, cholesterol feeding significantly increased serum triglyceride levels from baseline (Figure 14B). However, after two weeks of cholesterol withdrawal, triglyceride levels normalized. Longer durations of cholesterol withdrawal resulted in further decreases in triglyceride levels until these stabilized below baseline.

1.3 Serum CRP profile

Four weeks of cholesterol-enriched diets significantly elevated serum CRP levels from baseline levels (Figure 14C). Circulating levels of CRP remained elevated until the 6th week of cholesterol withdrawal. Thereafter, an additional two weeks of cholesterol withdrawal significantly decreased CRP levels below baseline and these levels remained below baseline even with another sixteen weeks of cholesterol withdrawal from the diet (Figure 14C).

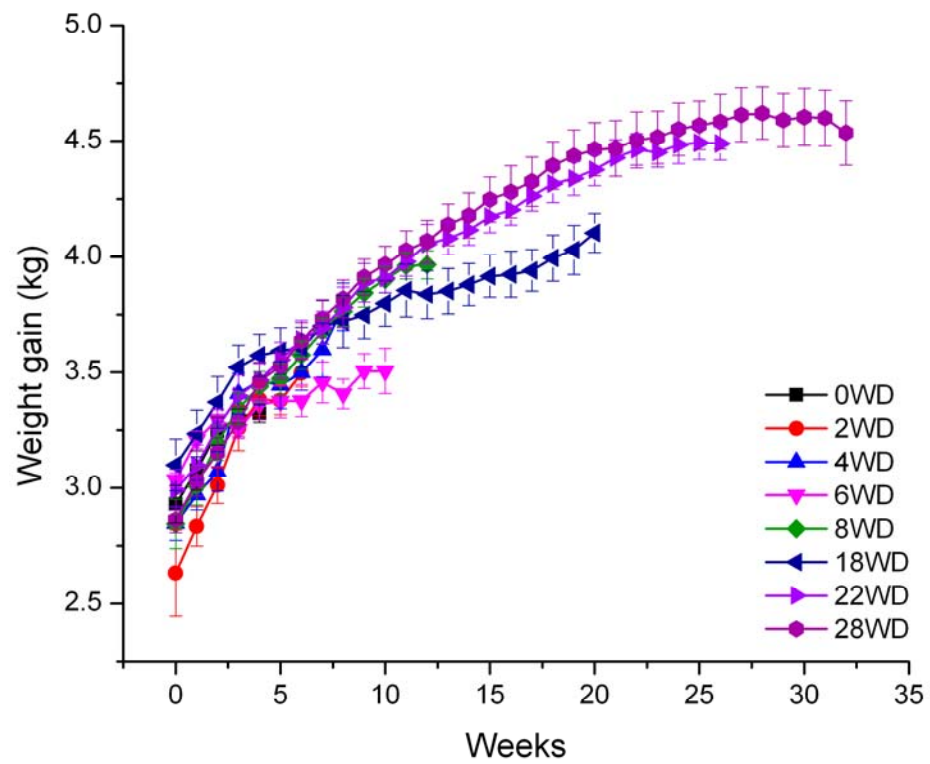


Figure 13. Average weight gain of animals given various duration of cholesterol withdrawal after 4 weeks of a high cholesterol diet. Values are mean \pm SE; n= 6-10

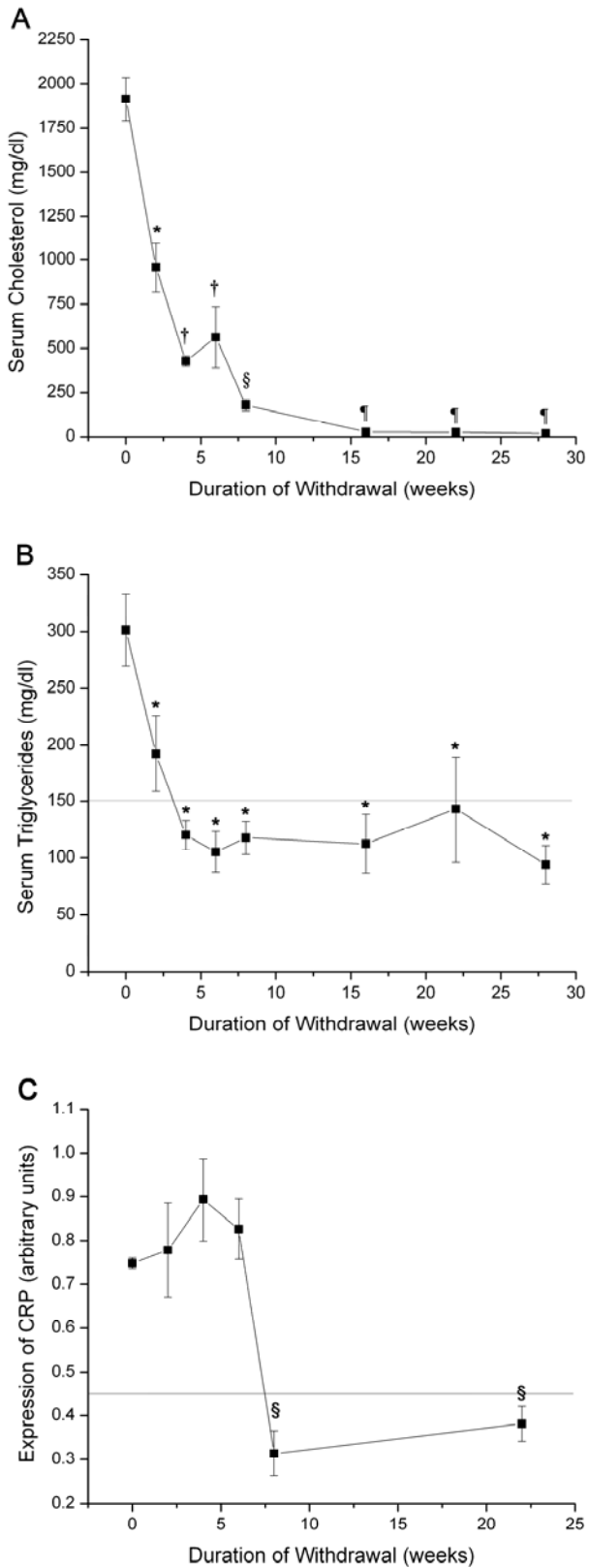


Figure 14. Changes in cholesterol (A), triglyceride (B) and C-reactive protein (C) concentrations within the plasma after prolonged withdrawal from cholesterol feeding. The grey line in B and C represents the levels in rabbits fed regular rabbit chow for 12 weeks. Values are mean \pm SE, n=6-10. *P < 0.05 vs 0WD; †P < 0.05 vs 0WD,2WD; ‡P < 0.05 vs 0WD,2WD, 4WD, 6WD; ¶P < 0.05 vs 0WD,2WD, 4WD, 6WD,8WD.

1.4 Effects of cholesterol withdrawal on lesion development

Atherosclerotic plaque formation began approximately at 4 weeks of cholesterol feeding and, thereafter, continued to progress even after the removal of cholesterol from the diets of these animals. Significant lesions were clearly visible on the aortic luminal surface. Eight weeks after cholesterol withdrawal, plaque growth had stabilized and did not progress further. Aortic lesion area began to show evidence of a gradual decrease after >8 weeks of maintenance on a regular rabbit chow (Figure 15).

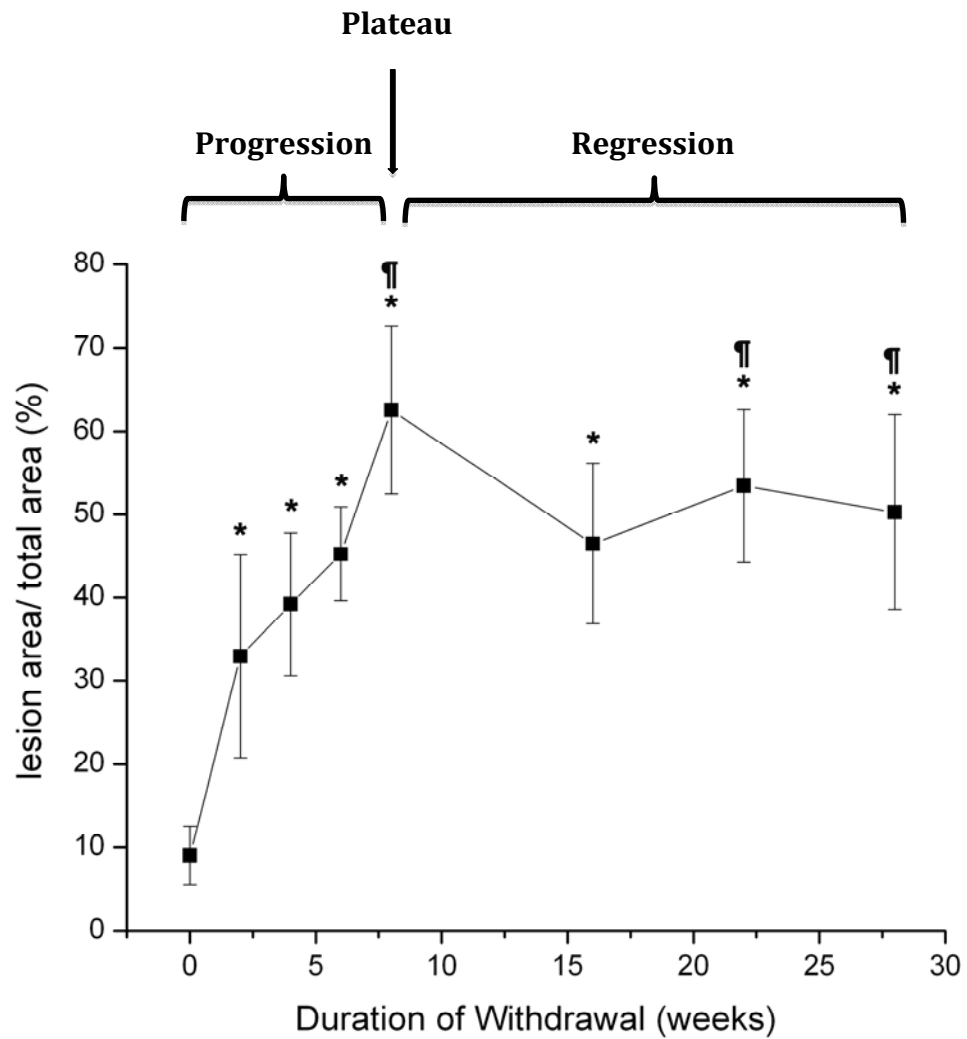


Figure 15. Progression, plateau and regression of aortic atherosclerotic lesion after prolonged withdrawal from cholesterol feeding. Values are means \pm SE; $n = 8 - 10$. ¶ $P < 0.05$ vs Group II.

2. *Effects of Flaxseed supplementation on plaque regression*

2.1 *Animal weight gain*

Weight gain did not differ significantly ($P > 0.05$) amongst all experimental groups (Figure 16), which was ultimately a reflection of the similar energy content of the different diets.

2.2 *Diet induced changes in lipid profiles*

The circulating cholesterol levels of Group II animals fed a regular rabbit chow after four weeks of cholesterol supplementation were significantly elevated, being nearly 10-fold higher than control (Figure 17A). After an additional 8 weeks of regular rabbit chow (Group III) or a 10% flaxseed-supplemented diet (Group IV), cholesterol levels returned to baseline (Figure 17A). These levels did not change with an additional 6 weeks of either diet (Group V and VI). In contrast, animals maintained on a regular diet for 12 weeks (Group I) had significantly decreased levels of serum triglyceride compared to animals that were fed 4 weeks of a high cholesterol diet followed by 8 weeks of cholesterol withdrawal (Group II). Longer withdrawal caused a progressive decrease of these levels; however, these decreases did not reach statistical significance (Figure 17 B).

Total serum fatty acids (TFA) were quantified and resolved (Table 5). Although the total TFA levels did not significantly differ between the experimental groups, there were notable differences in the serum fatty acid composition. Even after 8 weeks of withdrawal from dietary cholesterol, Group II animals exhibited serum levels of palmitoleic acid (C16:1), vaccenic acid (C18:1) and oleic acid (C18:1 O) that remained significantly increased compared to control animals (Table 5). A longer period on both regular rabbit chow and flax-supplementation was able to significantly decrease the

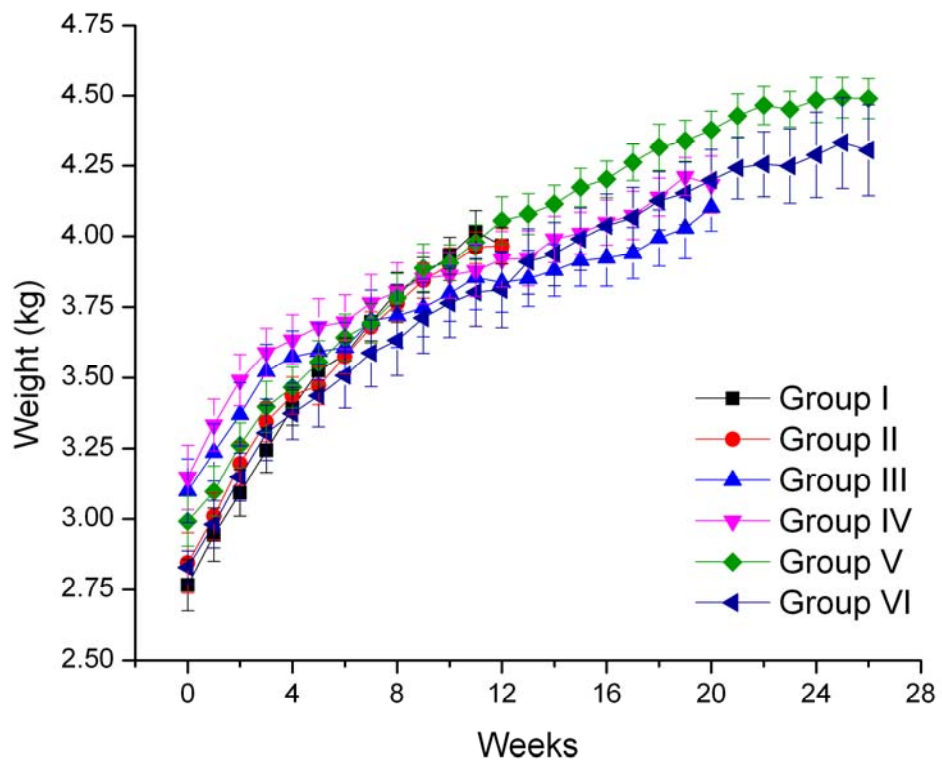


Figure 16. Average weight gain of animals in various treatment groups. Values are mean \pm SE; n=8-10

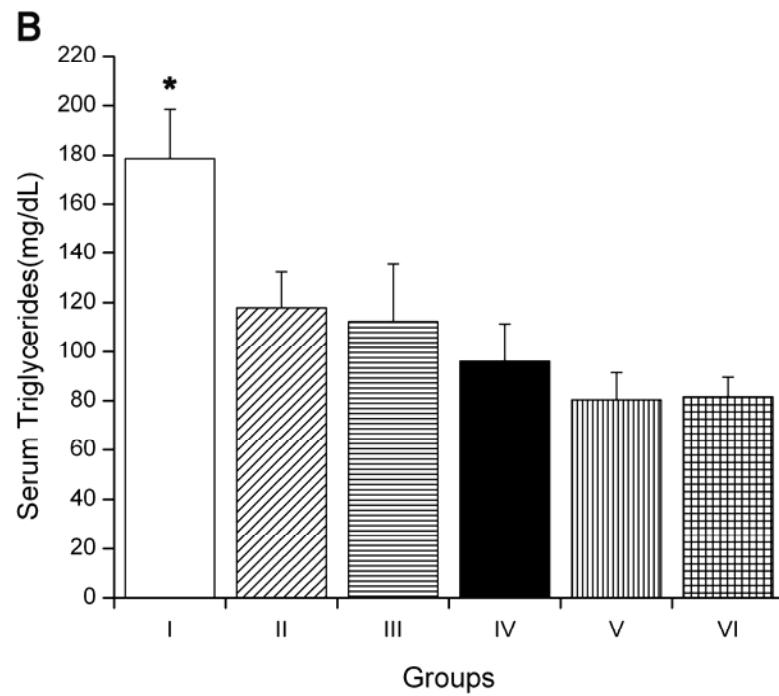
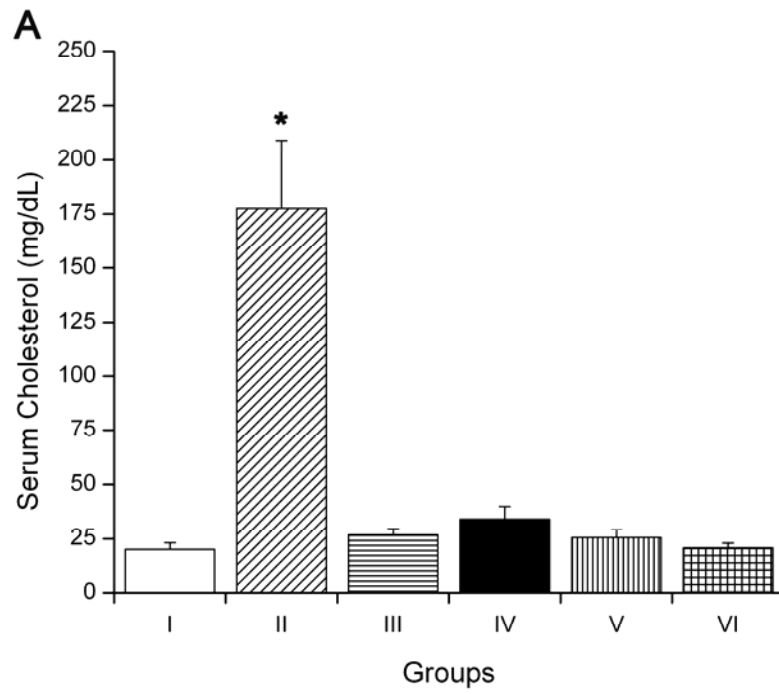


Figure 17. Serum cholesterol (A) and triglyceride (B) concentration in rabbits following the completion of respective dietary treatments. Values are mean \pm SE; n-8-10. * P<0.05 vs all other groups.

Table 5. Serum fatty acid concentrations of rabbits after completion of dietary regimens.

	Group I	Group II	Group III	Group IV	Group V	Group VI
14:0	17.0±4.3	33.2±9.1	14.8±3.5	21.5±2.7	22.3±2.7	15.1±3.1
16:0	244.6±18.6	414.0±86.9	181.5±18.7*	267.4±67.0	212.1±24.4*	185.1±12.4*
18:0	72.3±6.31	121.1±26.0	64.6±5.1	99.3±16.5	70.2±7.1	85.5±6.4
20:0	t	t	t	t	t	t
22:0	t	1.3±1.1	t	t	t	t
24:0	t	t	t	t	t	t
16:1	42.4±5.8*	105.6±25.6	31.8±6.50*	30.8±5.5*	49.7±7.9*	31.5±4.0*
18:1 O	306.2±24.3*	575.6±115.3	219.7±16.7*	269.3±45.4*	249.8±27.8*	200.3±20.1*
18:1	21.2±4.5*	59.2±12.1	19.6±3.5*	19.2±5.0*	20.9±4.3*	10.5±2.5*
20:1	t	5.9±3.6	t	1.6±1.6	t	t
22:1	t	t	t	t	t	t
24:1	t	t	t	1.6±1.6	t	t
18:2(ω-6)	211.0±16.2	340.4±72.7	197.9±15.5	291.6±66.1	212.2±23.8	227.5±15.4
18:3(ω-6)	t	t	t	t	t	t
20:2(ω-6)	t	1.9±1.1	t	t	t	t
20:3(ω-6)	t	1.5±1.5	t	t	t	t
20:4(ω-6)	18.4±1.2	37.6±5.9	18.4±1.2*	26.3±9.6	17.3±3.40*	18.4±3.0*
18:3(ω-3)	36.7±3.5†	49.7±12.5†	31.0±2.7†	132.2±24.9	40.3±6.8†	170.1±18.1
20:3(ω-3)	t	t	t	5.2±3.3	t	t
20:5(ω-3)	t	t	t	t	t	t
22:5(ω-3)	t	1.1±1.0	t	9.0±7.9	t	t
22:6(ω-3)	t	t	t	t	t	t
ω-6/ω-3	6.3±0.3†	6.7±0.9†	6.4±0.3†	2.7±1.2	5.8±0.5†	1.5±0.2
TFA	970.0±84.6	1748.0±374.5	779.4±73.4	1176.1±258	895.7±109.1	945.4±86.5

Values are mean ± SE, in µg/ml. Samples were obtained from animals after following their dietary regimens (n = 4-7). t, trace amounts present (<1 µg/100 mg fatty acids); TFA total fatty acids (mg fatty acid in 1 ml of plasma). *P < 0.05 vs Group II. †P < 0.05 vs Group IV and VI.

levels of these fatty acids. Palmitic acid (C16:0) and AA (C20:4 ω -6) levels significantly decreased with more time on regular rabbit chow and after sixteen weeks of flaxseed supplementation. Significant decreases in (C16:0) and (C20:4 ω -6) were not achieved after 8 weeks of flaxseed-supplementation (Group III). The addition of flaxseed into diets significantly increased serum ALA levels (Table 5). Despite the increases in ALA, its derivatives EPA (C20:5 ω -3), DPA (C22:4 ω -3, and DHA (C22:5 ω -3) were only detected in trace amounts. Flaxseed supplementation also significantly decreased the ω -6/ ω -3 PUFA ratio compared to all other experimental groups (Table 5).

2.3 *Serum C-reactive protein profile*

Even with an additional 14 weeks of regular rabbit chow or flaxseed-supplementation, serum CRP levels did not change (Figure 18). Moreover, the CRP levels were the same as the animals that were maintained on regular rabbit chow for 12 weeks (Group I).

2.3 *Effects of dietary intervention on platelet aggregation*

The various dietary regimens did not affect either the rate or the amount of platelet aggregation (Figures 19-22).

2.4 *Effects of dietary interventions on vascular function*

Vascular function was first evaluated with agonist-mediated vasoconstriction. There were no significant differences in KCl-induced vasoconstriction amongst the various groups (Figure 23A). Eight weeks of dietary withdrawal of cholesterol had no effect on the contractile response of aortic rings to NE; however, there was a trend towards attenuated contractility that did not achieve significance (Figure 23B). Neither a flaxseed-supplemented nor a regular diet was able to significantly improve responsive-

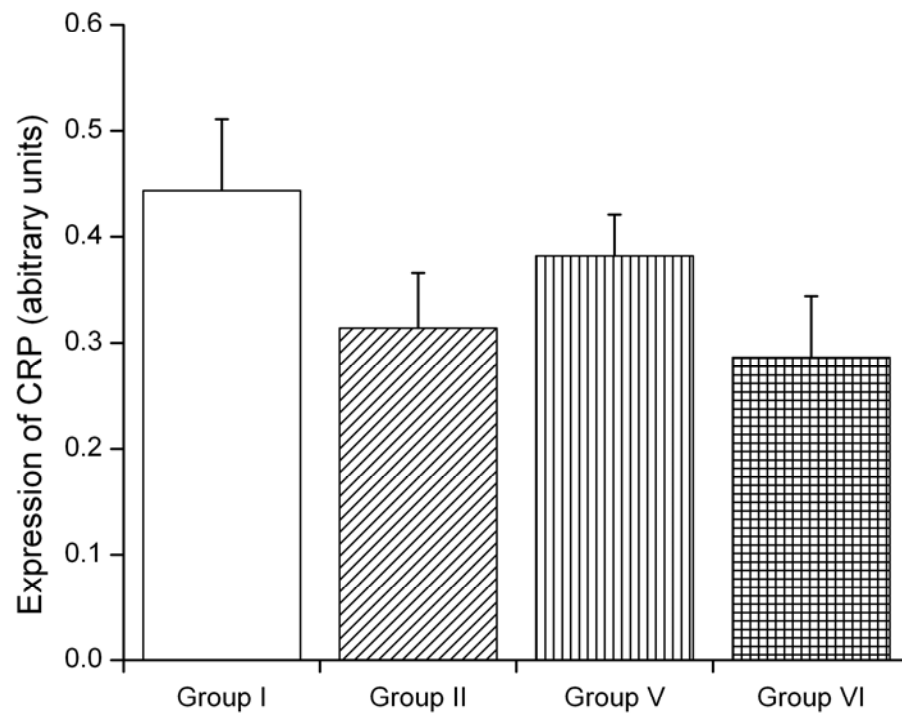


Figure 18. Expression of CRP in the serum of animals given different dietary regimens. Values mean \pm SE; n=5-8

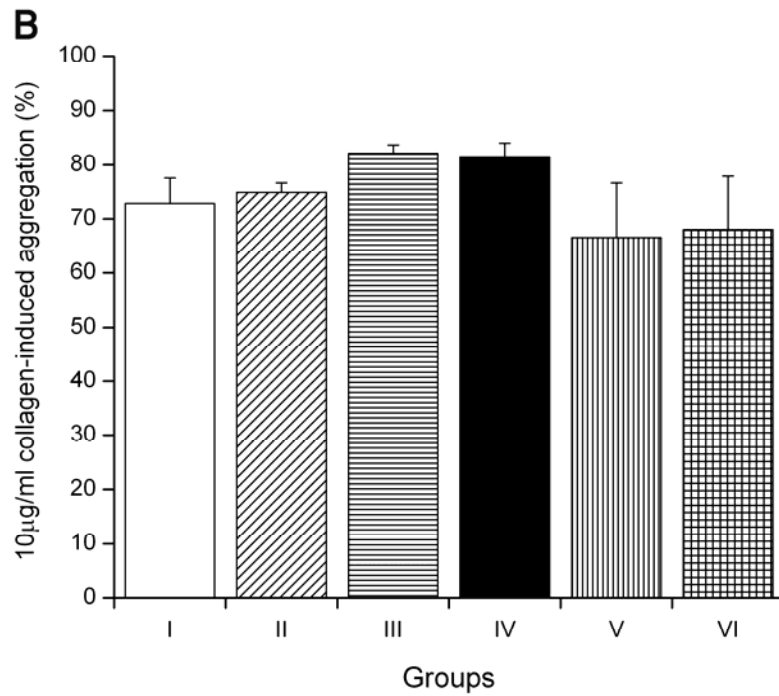
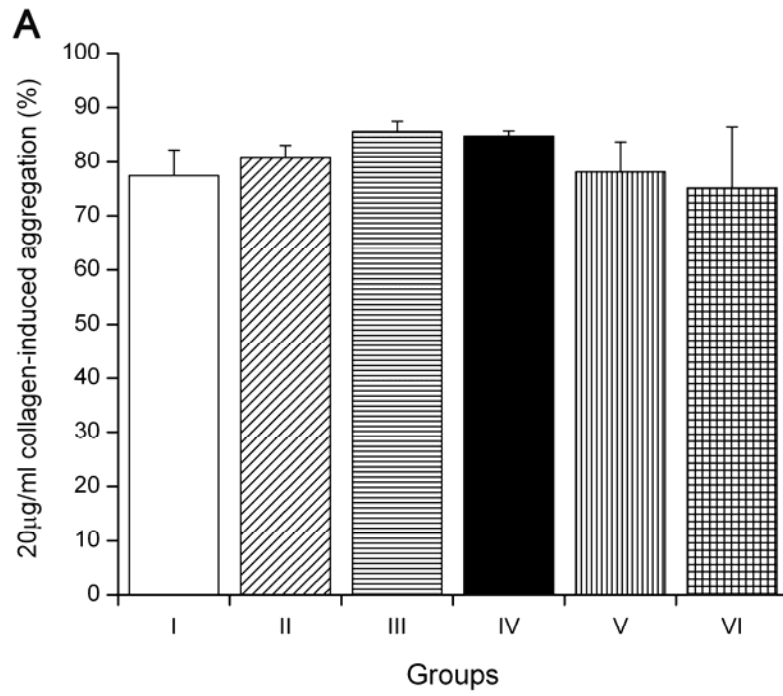


Figure 19. Total platelet aggregation of plasma taken from group I-VI animals after completion of dietary treatment. Platelet aggregation was initiated by 20µg/ml and 10µg/ml of collagen. Values mean \pm SE; n=7-10

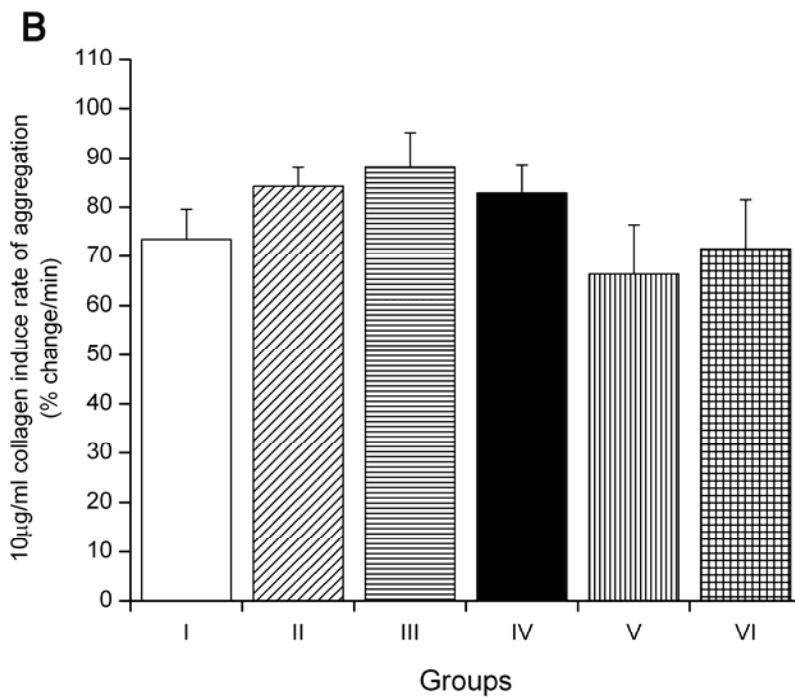
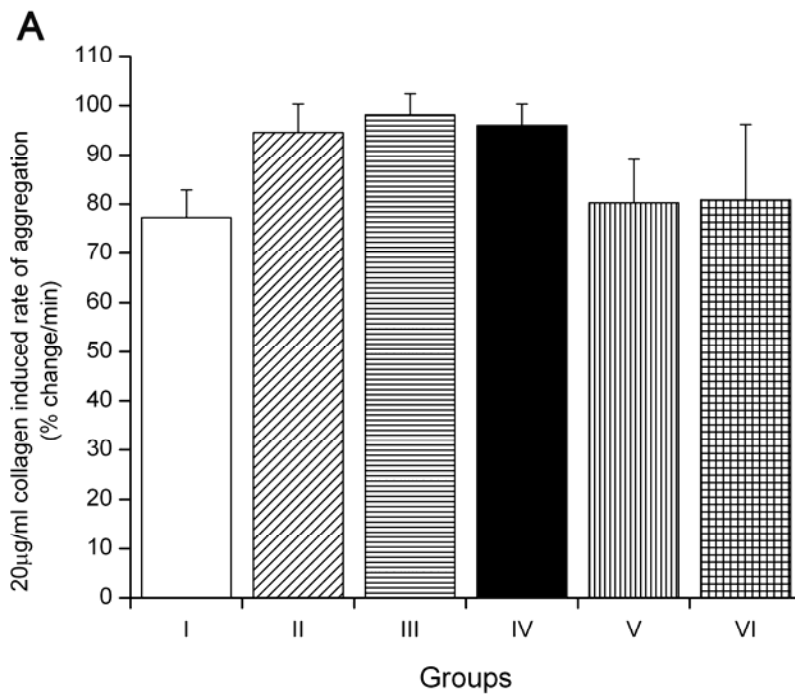


Figure 20. Rate of platelet aggregation as a function of dietary regimens in rabbits. Aggregation was induced by 20 µg/ml and 10 µg/ml of collagen. Values are mean ± SE; n=7-10.

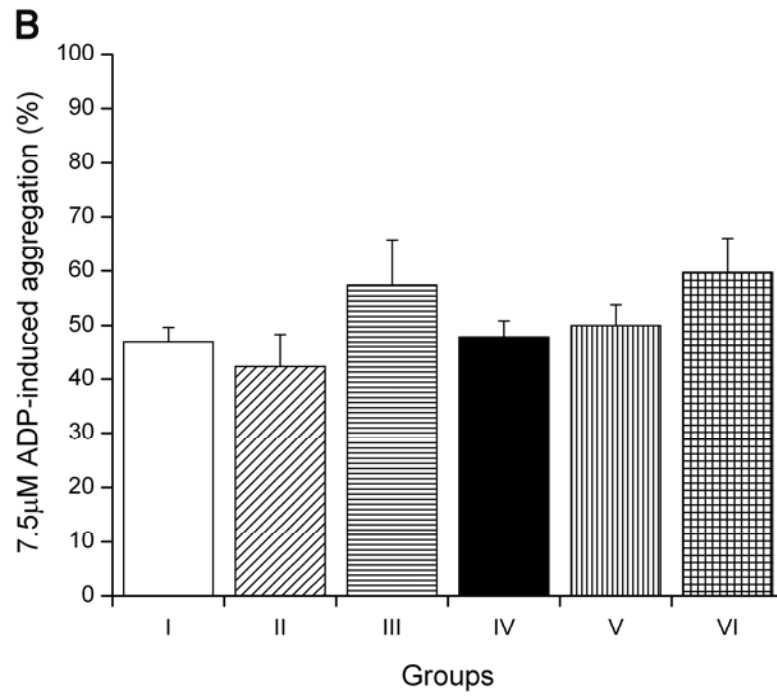
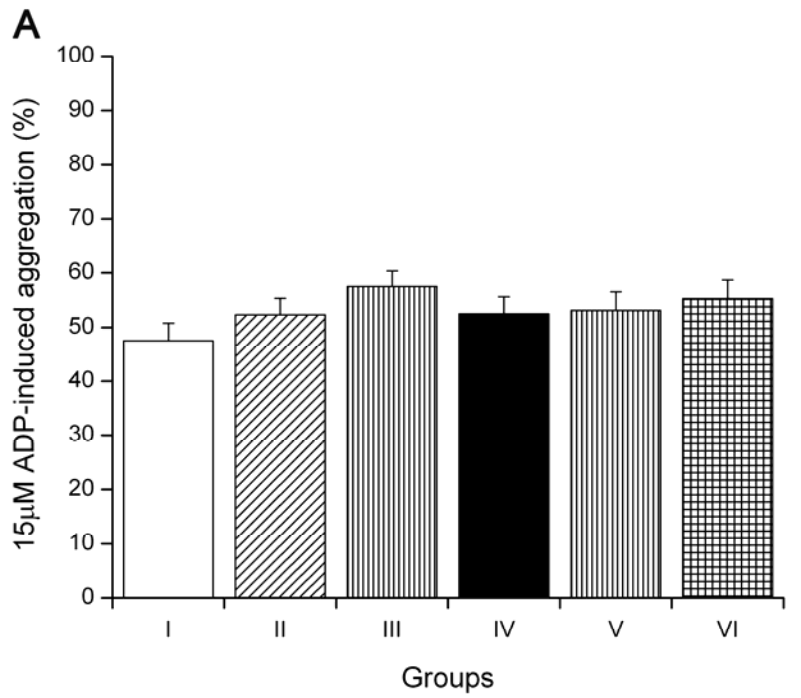


Figure 21. Total platelet aggregation of plasma taken from group I-VI animals after completion of dietary treatment. Platelet aggregation was initiated by 15µM and 7.5µM of ADP. Values mean ± SE; n=7-10

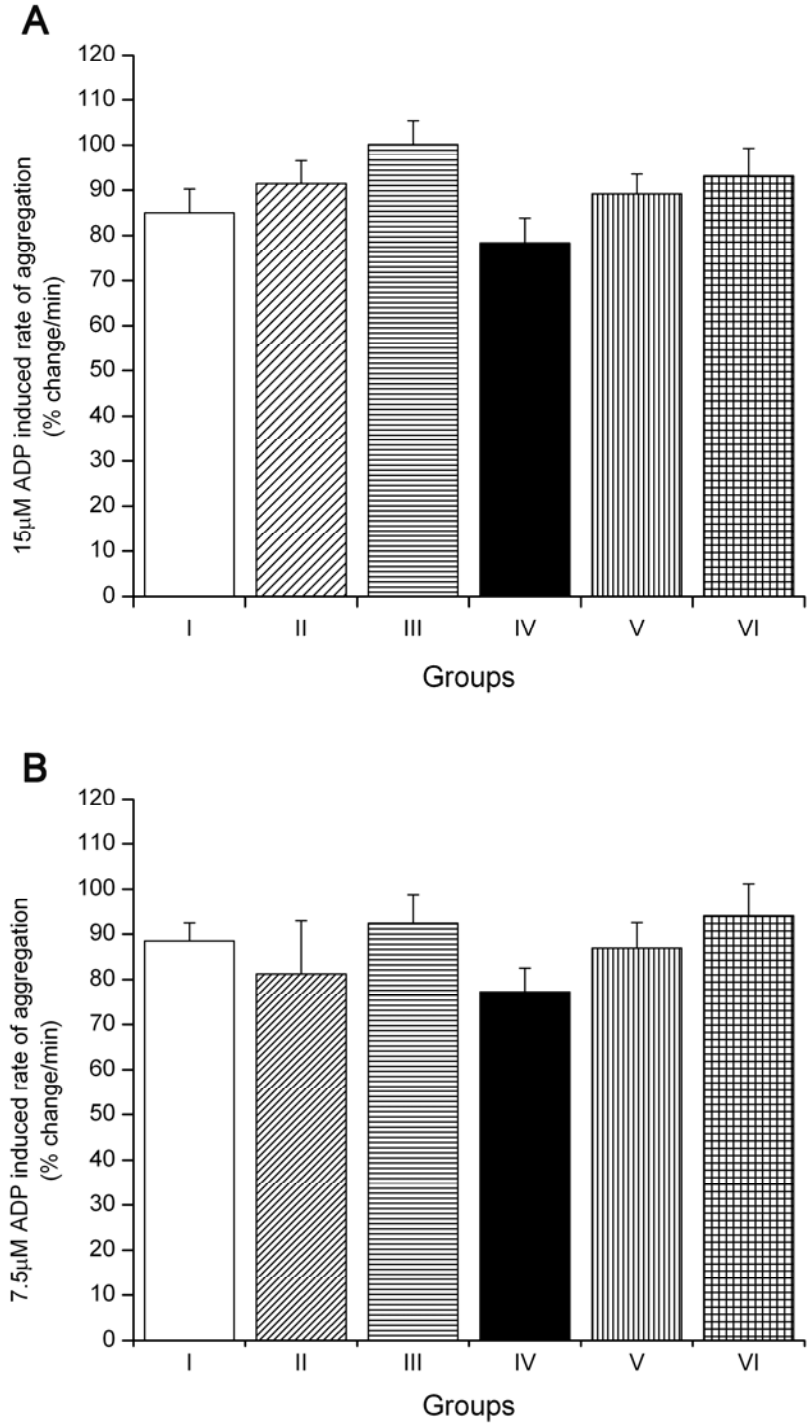


Figure 22. Rate of platelet aggregation as a function of dietary regimens in rabbits. Aggregation was induced by 15µM and 7.5µM of ADP. Values are mean ± SE; n=7-10.

ness to NE.

The responsiveness of aortic rings to vasodilatory mediators was elucidated after pre-contraction with 10^{-6} M NE. Even after 8 weeks of withdrawal from cholesterol feeding, aortic rings taken from Group II animals showed substantial impairment in their response to doses of Ach, which became significant at high concentrations of Ach (10^{-6} M and 10^{-5} ; $p=0.057$) (Figure 24A). Although both interventions caused notable improvements in the response to Ach, their recovery in respond to acetylcholine did not reach statistical significance. The tension produced by aortic rings from Group III when stimulated to relax with 10^{-7} M SNP was significantly less than Group II animals. Despite this exception, there were no other significant differences between groups in the extent of aortic relaxation caused by SNP (Figure 24B).

2.6 *Effects of dietary regimens on atherosclerosis*

Plaque formation was not visible on the aortic surface of control animals (Figure 25). The plaques were only beginning to become visible slightly four weeks after the initiation of cholesterol feeding. Plaque progression was more rapid after the withdrawal of cholesterol from the diet. Even with the addition of a regular diet, plaques progressed until eight weeks after cholesterol withdrawal. At eight weeks after the withdrawal of cholesterol from the diet, extensive atherosclerotic plaque development was evident on the luminal surface of these animals (Figure 25). After this period of time, the plaque progression stabilized and did not exhibit further increases in lesion area (Figure 25). The extent of lesion area substantially decreased (~40%) after an additional 14 weeks on the flaxseed-supplementation diet (Figure 25). Although a trend was evident, this association ($P= 0.154$) did not reach statistical significance ($P> 0.05$) relative to the cont-

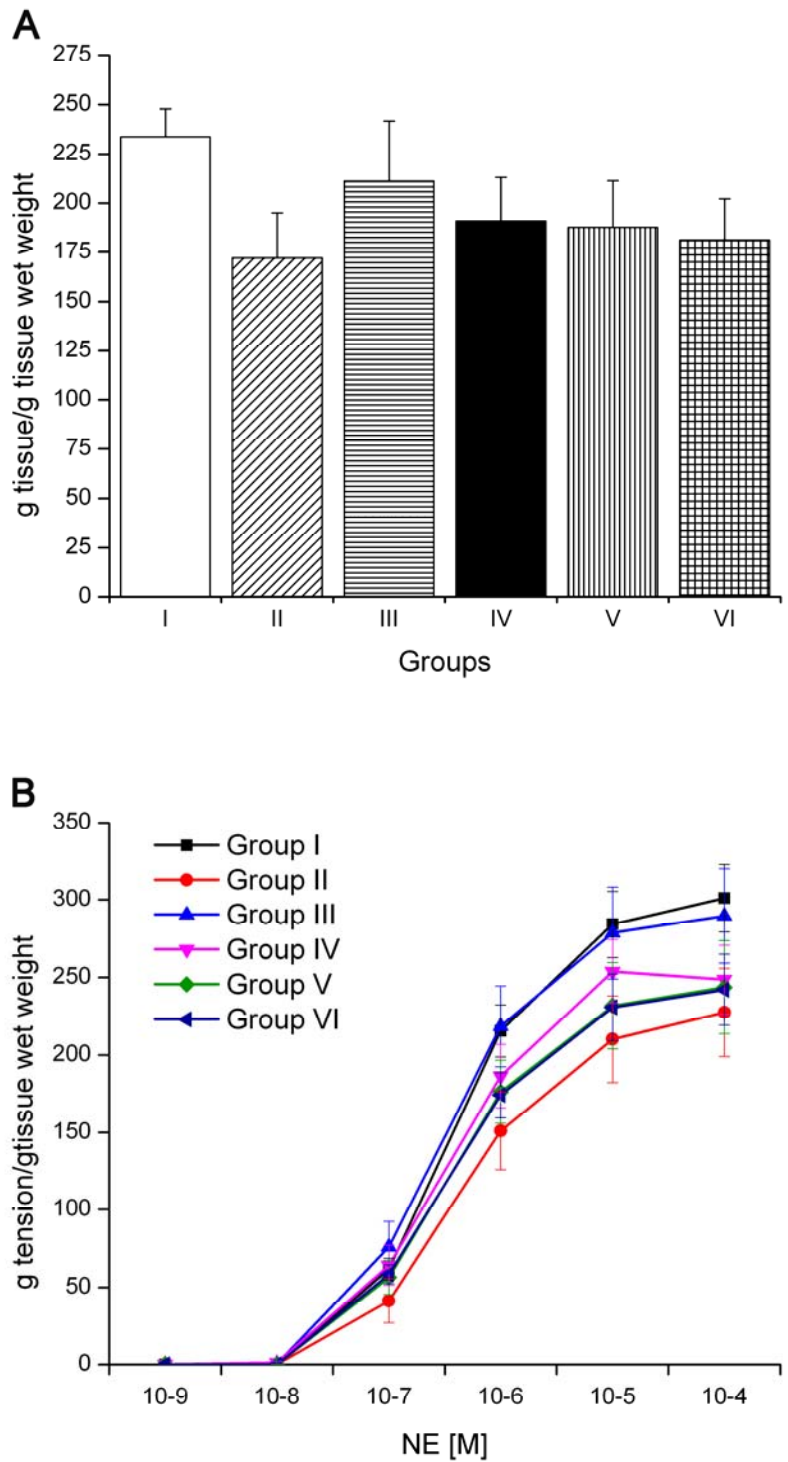


Figure 23. Contractile response to 47mM KCl (A) and increasing doses of norepinephrine [NE] (B) of proximal aortic rings taken from group I-VI animals following dietary treatments. Values are mean \pm SE, n=7-9

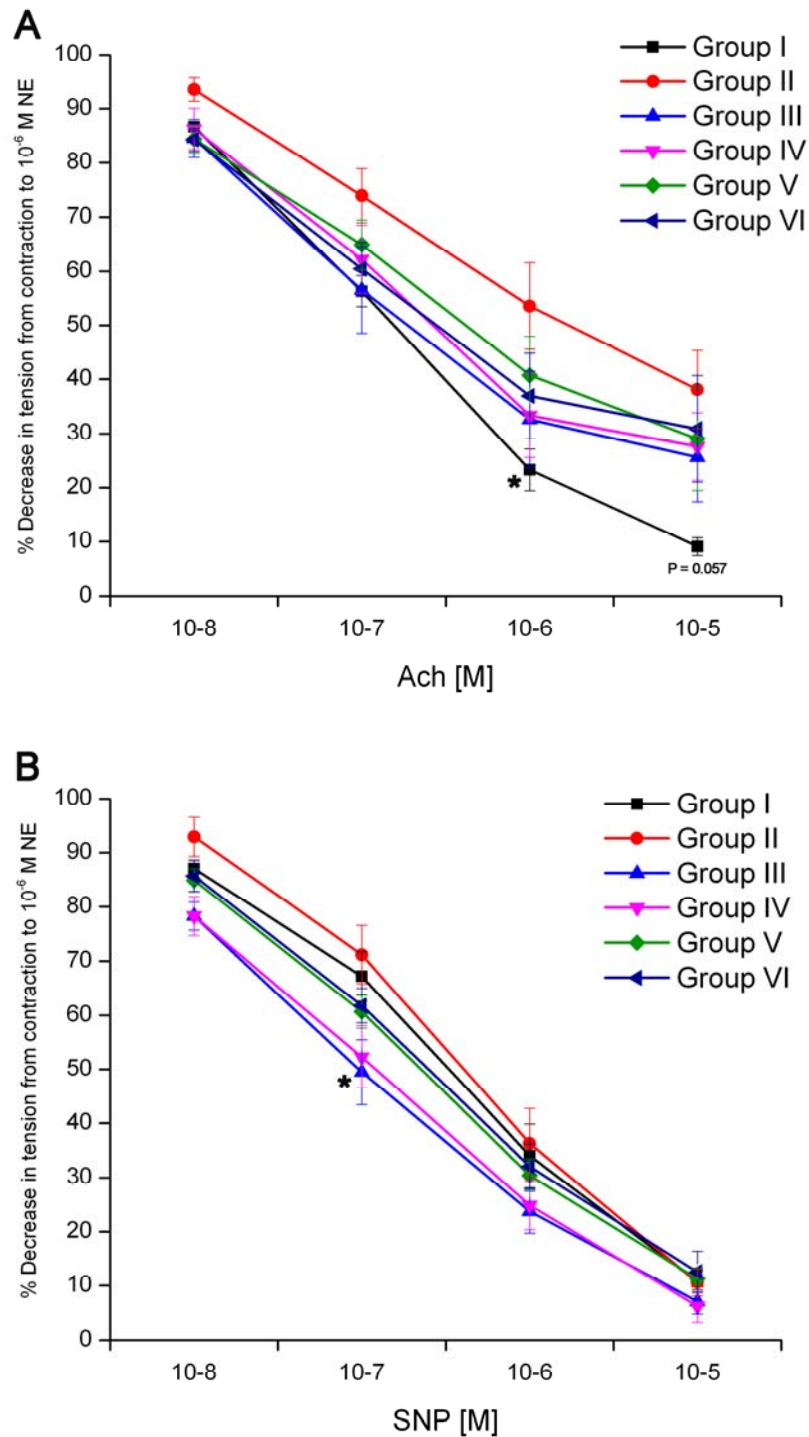


Figure 24. Acetylcholine [Ach] (A) or sodium nitroprusside [SNP] (B) induced relaxation in aortic rings isolated from animals on different dietary regimen after precontraction with 10^{-6} NE. Results are presented as percent decrease from maximal contraction with 10^{-6} NE. Values are mean \pm SE, n=6-9. *P<0.05 vs Group II.

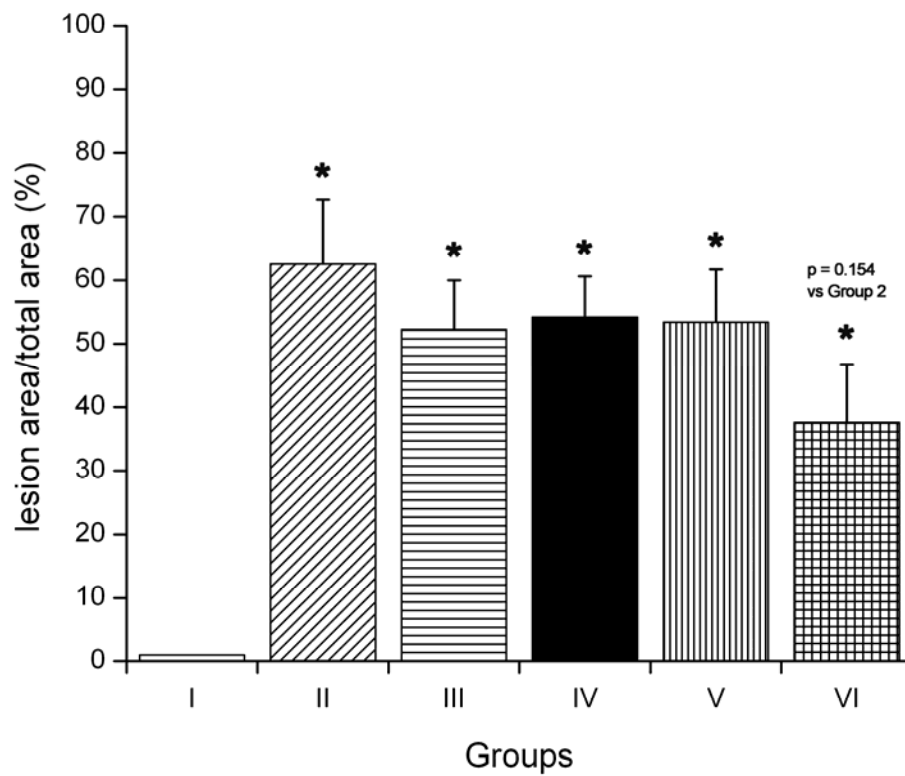


Figure 25. Extent of aortic atherosclerotic lesions following dietary regimens. Group I animals did not develop atherosclerotic lesions within the aorta. Values are mean \pm SE; n-8-10. *P<0.05 vs Group I

ol diet group (Group I).

2.7 *Diet induced changes in aortic lipid content*

Cross-sectional analyses revealed that even after eight weeks of cholesterol withdrawal significant amounts of Oil Red O staining were detected in the aorta. Nevertheless, additional time on the regular rabbit chow or flaxseed-supplemented diet was unable to decrease these levels (Figure 26). In addition, there were no significant differences in the amounts of Oil Red O staining between proximal, medial and distal aspects of rabbit aortas (Figure 26).

In contrast, significant changes were observed in total aortic cholesterol content. After 8 weeks of cholesterol withdrawal, the cholesterol concentration within the aorta of Group II animals remained significantly (10-fold) increased (Figure 27). Additional time on either intervention decreased these levels, which subsequently achieved significance when further extended (Figure 27).

Compared to the previously described serum levels, more robust diet-induced alterations in the levels and distributions of fatty acids were observed in aortic tissue (Table 6). There were no significant changes in TFA content between groups. Regardless, 8 weeks of cholesterol withdrawal significantly increased the ω -6/ ω -3 ratio as well as the levels of various long chain fatty acids including: stearic acid (C18:0), vaccenic acid, eucic acid (C20:1), eicosadienoic acid (C20:2 ω -6), and dihomo-gamma-linolenic acid (C20:3 ω -6). These levels were significantly reduced with an additional fourteen weeks on the regular rabbit diet. Moreover, this same diet significantly reduced levels of LA (C18:2 ω -6). Dietary supplementation with flaxseed caused significant increases in ALA and eicosatrienoic acid (C20:3 ω -3) levels as well as significantly

decreased the ω -6/ ω -3 PUFA ratio in the aortic tissues. Likewise, flaxseed supplementation was able to increase levels of DPA relative to Group I and V animals. However, only after fourteen weeks of flaxseed supplementations did DHA levels significantly decrease.

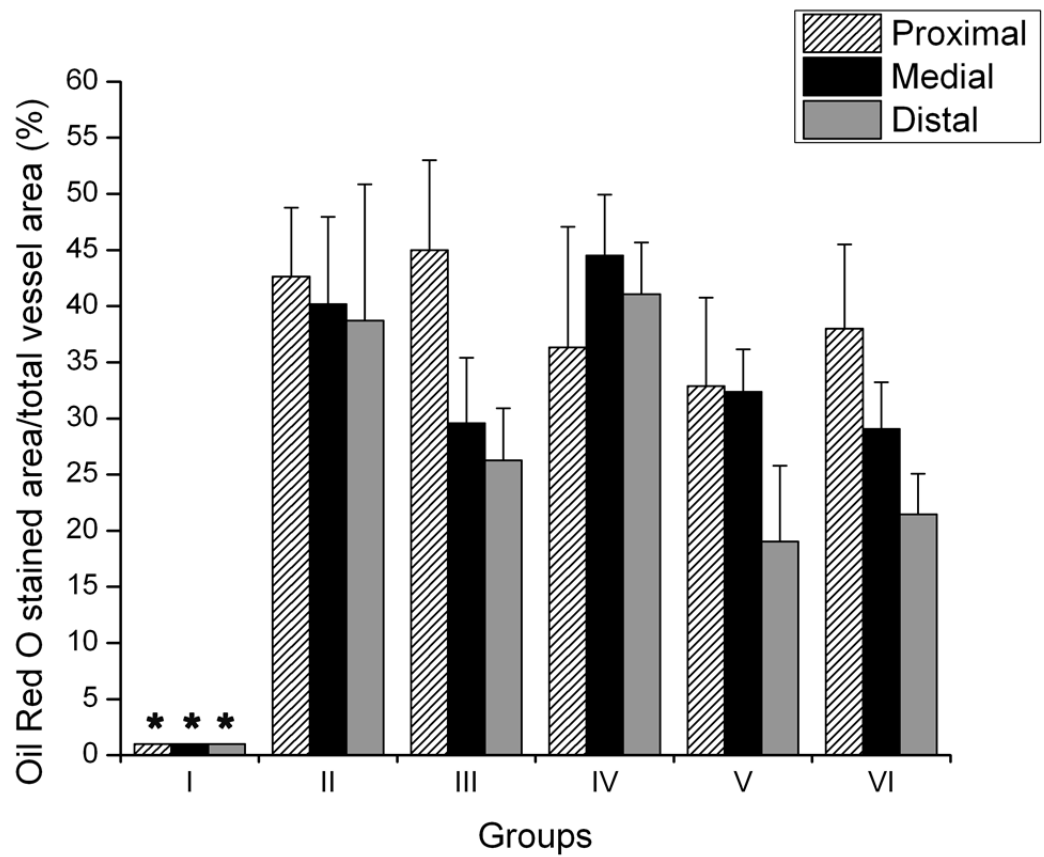


Figure 26. Percent area of Oil Red O staining detected within proximal, medial and distal aortic cross-sections of group I-VI rabbits after completion of dietary treatments. Values are mean \pm SE; n=3-5. * P<0.05 vs Groups II-VI

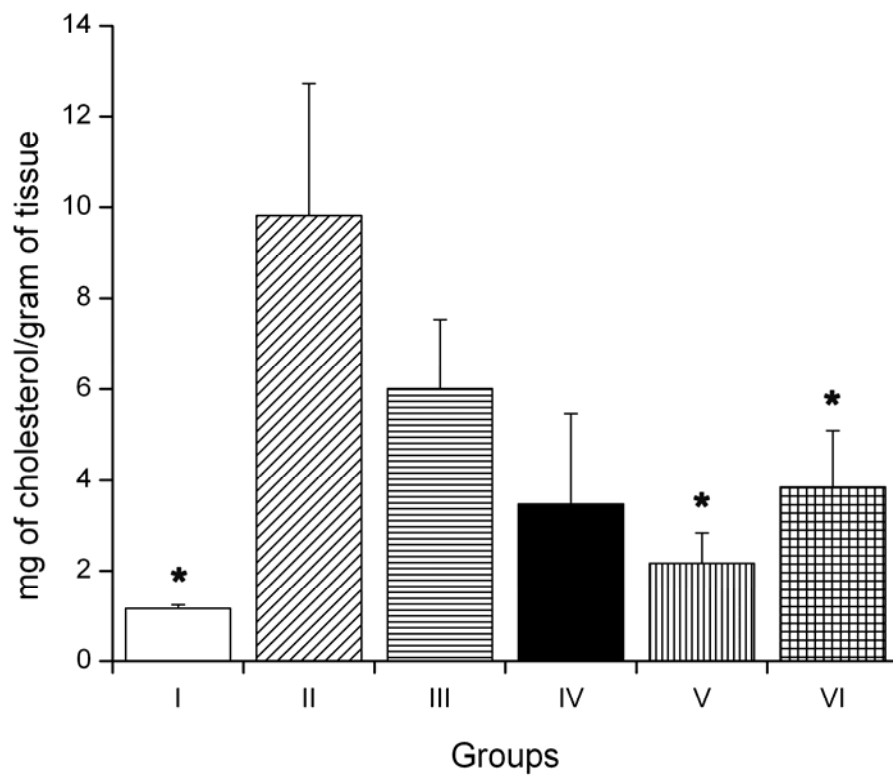


Figure 27. Total aortic cholesterol content of animals after completion of dietary treatments. Values are mean \pm SE; n=3-5. * P<0.05 vs Group II

Table 6. Aortic fatty acid concentrations of rabbits after completion of dietary regimens.

	Group I	Group II	Group III	Group IV	Group V	Group VI
14:0	113.9±16.3	130.1±23.7	116.8±29.8	97.2±4.1	77.7±4.3	80.1±17.0
16:0	1772.3±240.9	2432.7±291.3	1940.4±398.9	1778.6±1098	1342.5±132.7	1368.5±138.4
18:0	678.5±72.3*	883.6±134.5	680.4±108.4	736.6±54.1	530.1±40.1*	663.8±75.7
20:0	35.9±5.3	43.0±7.9	34.3±4.5	40.6±3.0	32.4±3.5	39.4±2.5
22:0	41.9±6.2	44.0±6.5	31.5±4.5	40.5±2.3	35.6±3.1	44.4±2.7
24:0	46.0±7.2	60.1±10.0	38.7±3.8	50.6±4.7	4.2±4.0	55.1±5.0
16:1	239.2±33.7	321.3±69.7	38.7±5.2	201.8±24.3	175.1±10.5	139.8±23.8
18:1 O	1763.5±178.4	4610.5±1047.8	3429.9±973.2	2905.4±528.8	1740.6±196.0	2460.6±581.7
18:1	152.1±11.0*	638.5±169.3	348.6±109.0	280.4±49.2	197.6±38.6*	274.2±97.0
20:1	23.9±2.7*	157.0±49.1	85.6±32.4	59.4±16.7	37.6±7.9*	61.5±24.7
22:1	t	t	t	40.5±2.3	t	t
24:1	16.6±3.0	123.1±42.5	78.2±20.3	82.5±34.1	49.0±17.4	70.2±37.3
18:2(ω-6)	1050.2±117.6	1898.7±301.9	1657.7±369.8	1544.8±154.9	887.2±78.3*	1051.0±112.1
18:3(ω-6)	t	t	2.9±1.8	2.8±2.8	t	t
20:2(ω-6)	17.5±1.8*	205±66.3	98.3±37.1	76.1±25.7	34.9±8.6	72.2±28.1
20:3(ω-6)	13.5±2.0*	83.6±23.4	54.7±14.6	45.0±15.2	25.8±8.5	43.8±14.3
20:4(ω-6)	614.0±94.9	825.8±109.9	634.8±101.4	697.1±30.8	614.2±75.4	726.2±67.3
18:3(ω-3)	174.4±20.4†¶	243.9±25.7†¶	244±51.7†¶	679.5±103.9	130.0±13.6†¶	432.4±95.9†
20:3(ω-3)	t	41.6±18.4	19.2±8.8	50.2±12.9	3.9±1.6	47.9±23.0
20:5(ω-3)	t	6.3±6.3†¶	4.6±2.9†¶	33.7±3.4	2.3±1.4†¶	30.0±11.8
22:5(ω-3)	68.5±10.8†¶	78.7±16.7	75.8±13.6	145.4±16.6	60.5±9.0†¶	144.4±28.9
22:6(ω-3)	12.9±2.4	13.4±7.0¶	12.5±5.0¶	28.3±6.1	16.3±2.5¶	39.4±7.4
ω-6/ω-3	6.6±0.4*†¶	9.6±0.8	6.9±0.2*†¶	2.6±0.2*	7.4±0.5*†¶	3.0±0.5*
TFA	6835.7±827.9	12839.6±2427.7	9870.7±2381.9	9617.1±1206.0	6035.6±657	7845.0±1395

Values are mean ± SE, in µg/g. Samples were obtained from animals after following their dietary regimens (n = 4-7). t, trace amounts present (< µl/100 mg fatty acids); TFA total fatty acids (mg fatty acid in 1 g of aorta). *P<0.05 vs Group II; †P<0.05 vs Group IV; ¶P<0.05 vs Group IV and VI.

Discussion

Rabbits represent an ideal model with which to study the effects of nutritional interventions on atherosclerotic plaque dynamics because they 1) do not require genetic manipulation to create plaques, 2) develop atherosclerotic plaques after dietary cholesterol supplementation, and 3) have similar lipoprotein transport mechanisms to humans [265-268]. In the present study, we have observed a clear dissociation of circulating cholesterol levels from atherosclerotic plaque formation. As hypercholesterolemia was gradually resolving during the withdrawal period from cholesterol, aortic plaques continued to progress (Figure 14 and 16). Concurrently, triglyceride levels decreased below baseline levels, a common age-dependent phenomenon observed in rabbits [6, 18, 22, 27]. The continued progression of atherosclerotic lesions despite the marked decrease in circulating cholesterol may seem paradoxical. However, cholesterol levels remained ten-fold higher than normal when plaque progression ceased at 8 weeks post cholesterol supplementation to the diet (Figure 14A). Reidmüller et al. [27] have also reported that serum cholesterol levels were ten-fold higher than baseline when the highest lesion cholesterol and macrophage/foam cell content was observed during cholesterol withdrawal. Thus, even during cholesterol withdrawal, circulating cholesterol levels remain high enough to facilitate cholesterol deposition and foam cell accumulation in growing atherosclerotic plaques. Additionally, even after withdrawal from cholesterol, CRP levels continued to increase in conjunction with plaque progression (Figure 14 C). Similarly, Yu et al. [269] recently confirmed that plasma CRP levels are associated with the formation and progression of atherosclerotic lesions in rabbits. Thus, despite the declining levels of circulating cholesterol, the high

level of systemic inflammation may likewise aid in the progression of plaques. These observations may invalidate any conclusions about plaque regression in studies that initiated their intervention prior to the stabilization of the plaque [18-22]. Premature initiation of a nutritional intervention after cholesterol supplementation to the diet will only determine the ability of a food to slow plaque progression rather than its ability to induce plaque regression. Our data also demonstrate the importance of using an appropriate dietary control for nutritional interventions. Even if the nutritional interventions were initiated after stabilization of plaque development, without an adequate experimental control it would remain unclear whether a decrease in lesion area was due to the intervention or the mild regression that normally occurs when rabbits are maintained on a regular diet after plaque development plateau is achieved.

The present study demonstrates that the deleterious effects of cholesterol feeding on contractile function still persisted even after eight weeks of cholesterol withdrawal (Figure 23). Regardless, this trend did not achieve statistical significance, neither with NE-induced contraction nor KCl-induced contraction.

Prolonged hypercholesterolemia has been shown to impair acetylcholine-induced endothelial-dependent vasodilation in rabbits [265, 267, 268, 270]. We now demonstrate for the first time that the deleterious effects of cholesterol feeding on contractile function persist even eight weeks after the withdrawal of cholesterol from the diet. Pro-inflammatory and pro-atherogenic environments, caused by hypercholesterolemia, promote endothelial dysfunction [32, 35, 53, 54]. Since we could not detect any changes in the relaxation capacity of any of the vessels in response to NO delivered from SNP, the changes in relaxation observed are likely due to an endothelium-dependent decrease in

the bioavailability of NO [271, 272]. However, as the intimal diameter increases during atherosclerosis, the diffusion limit of NO (30-100 μm) is exceeded, also diminishing the amount that can reach the tunica media [273-275]. Both regular rabbit chow and flaxseed feeding restored endothelial-mediated vasodilation to the same extent (Figure 24). Riezebez et al. [276] have also shown that restoration of endothelial function occurred irrespective of pharmaceutical treatments with ramipril and isradipine. The observed improvements are likely the result of normal wound healing mechanisms that are also involved in atherosclerotic plaque regression including endothelial cell proliferation and replacement of dysfunctional cells with endothelial progenitor cells [9].

Although flaxseed supplementation yielded a trend towards an acceleration of atherosclerotic plaque regression, these findings did not achieve statistical significance ($P=0.154$). The biological differences in the anti-atherogenic responses of the rabbits to flaxseed may have induced enough variability that it did not achieve significance. Increasing the sample size, the dosage of flaxseed or the length of the dietary intervention may have allowed us to observe a statistically significant effect. The trend towards regression was accomplished without changes in systemic inflammation. This is the first study to show that as aortic lesions begin to regress, CRP levels rapidly decrease below baseline levels. In addition, these levels remain below baseline even after an additional 14 weeks of cholesterol withdrawal and flaxseed supplementation. Irrespective of the circulating levels of CRP, the expression of CRP within the lesion would be a more robust indicator of plaque inflammation.

Although the decreased lesion area seen after 14 weeks of dietary flaxseed intake is strong evidence of atherosclerotic plaque regression, both total aortic cholesterol

content and Oil red O stained tissue sections data did not show a trend towards regression (Figure 26 and 27). Aortic cholesterol content has been previously used to assess the degree of regression [27]. The small sample sizes in this study and the variability in lesion distribution and development between individual rabbits have diminished the strength of the relationship observed in the *en face* analysis. Additionally, the non-uniformity of lesion development has limited the use of Oil red O stained tissue sections to assess regression of atherosclerosis. Alternatively, tracking the development, progression and regression of individual animals with ultrasonography techniques such as B-mode ultrasound or intravascular ultrasound would be a substantial advantage over *en face* assessment of the lesion area within a group. Moreover, lesion markers of cell proliferation and inflammation such as PCNA, PPAR, mac-3, CRP and TNF- α should have been evaluated to assess and characterize cellular expression of these markers during regression [15, 269].

Caution must be taken when extrapolating these findings to humans. As previously mentioned, young rabbits do not develop plaques with complicated features. Rather, these lesions are fatty streaks that are consistent with Stary's type II lesions, which lack the clinical symptoms of Stary's type III-V lesions [86, 277, 278]. Thus, accelerated regression observed in young animals may be relevant to sub-clinical levels of atherosclerosis in humans. Additionally, observations in aortic lesions cannot simply be extrapolated to coronary atherosclerosis in rabbits. Coronary lesions require longer durations to develop and mainly contain VSMCs, while aortic lesions primarily consists of macrophage-derived foam cells [279]. However, it is possible to produce atherosclerotic lesions within rabbit that have many of the typical complex human

features [254, 258-260, 280]. Further studies are needed to determine whether flaxseed supplementation can accelerate regression of complicated lesions.

The literature shows a number of conflicting findings with respect to the effects of flaxseed and platelet aggregation for both human and animal studies [281-284]. In the present study, dietary flaxseed intake has no effect on the rate and magnitude of ADP and collagen-induced platelet aggregation. However, Dias and Taylor [284] showed that linseed (flax) oil significantly reduced collagen and thrombin-induced platelet aggregation in healthy rabbits. Flaxseed supplementation may not have the same effects in hypercholesterolemic or diseased animals. Of benefit would be an evaluation of platelet lipid content in animals with cholesterol-induced atherosclerosis, and, afterwards, the evaluation of nutritional interventions effects on the lipid content.

In summary, our data demonstrate a potential for flaxseed to accelerate the regression of atherosclerotic plaques in rabbits. Furthermore, since the flaxseed dosage utilized in this investigation is similar to that previously used in human studies [12, 243, 285, 286], our results suggest that a therapeutic dose of flaxseed may have the potential to accelerate the regression of sub-clinical, non-culprit lesions before they can potentially become more severe. However, further work to precisely identify the optimal concentrations of dietary flaxseed and/or the duration of the intervention may be required to clearly identify its beneficial actions. It is also important to acknowledge that focus should be on more global changes of the diet such as the Mediterranean diet rather than single dietary risk factors [239]. Thus, this study supports the incorporation of flaxseed into a healthy diet.

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