Diet-Induced Effects and the Potential for Cardiovascular Risk

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Abstract

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Cardiovascular disease remains the leading cause of death in America impacting the lives of millions. There are many modifiable risk factors that may help in the prevention and treatment of cardiovascular disease such as coronary artery disease or congestive heart failure. Proper diet and healthy weight management is a crucial factor in preventing disease. Dieting is a large component of American living, and the low-carbohydrate high-protein diet remains the most common form of weight loss. This thesis will investigate different types of diets and eating habits that can affect risk of developing cardiovascular disease.

An apolipoprotein E-deficient mouse model was used to investigate different diets and how they may develop cardiovascular disease, more specifically, atherosclerosis in the aorta and altered mitochondrial functioning in the heart. The diet of interest for this project, was a low-carbohydrate high-protein diet. This diet promotes an environment that causes a shift in cellular metabolism due to macronutrient imbalances. Past research has yet to address a possible link between low-carbohydrate high-protein diets and atherosclerosis and vascular smooth muscle cells, and mitochondrial functioning. This thesis contains data that will be of interest to both clinicians and patients, in terms of safe dieting and the potential for cardiovascular risk.

Table of Contents

List of Figures

I

Introduction

Theoretical Context

Cardiovascular Disease (CVD) is now the second leading cause of death in Canada [1]; the main contributor being ischemic heart disease and thus atherosclerosis [2]. With the rise of an overweight and obese population, proper weight maintenance is crucial for controlling risk factors for CVD and other conditions. Many people resort to exercise and strict dieting for weight loss and disease prevention, however not all diets have proven to be safe. Although effective for weight loss, the popular low-carbohydrate high-protein diets (LCHP) may be counterproductive for CVD prevention in that they may impose damaging effects to blood vessels. The implications of a low-carbohydrate high-protein diet will be explored in regards to potential effects on the development of atherosclerosis and vascular smooth muscle cell activity.

The Atherosclerotic Process and Pathway

Atherosclerosis is best described as a chronic inflammatory response to a vascular injury, involving many inflammatory cells, cytokines and adhesion molecules to form an atheroma [3-5]. An atheroma involves the deposition of cholesterol and other fatty substances as well as necrotic tissue within the intimal layer of an artery, rendering the lumen stenotic [6]. The artery is composed of three main layers: the intima, media, and adventitia (externa) as seen in Figure 1. On the innermost layer, the intima, lies a continuous layer of cells termed the "endothelium" that essentially separates the blood in the lumen from the rest

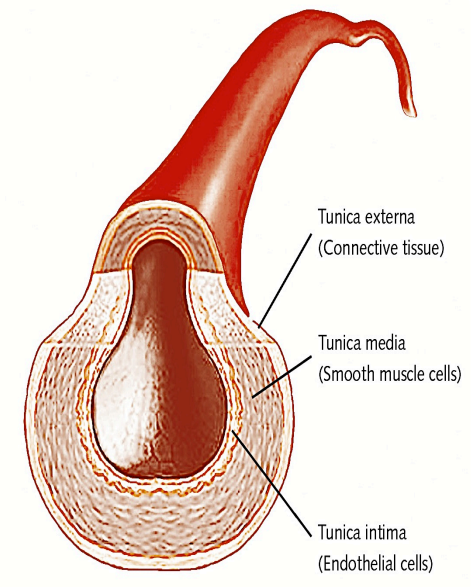

Figure 1: Layers of an artery [7]

of the vessel [8]. The endothelial layer composed of endothelial cells is involved in coagulation, inflammatory responses, and vascular tone via phosphorylation of endothelial nitric oxide synthase (eNOS) into nitric oxide (NO) [8,9]. The vascular injury present in this disease may be of physical or a biochemical origin that effectively alters the endothelium [10].

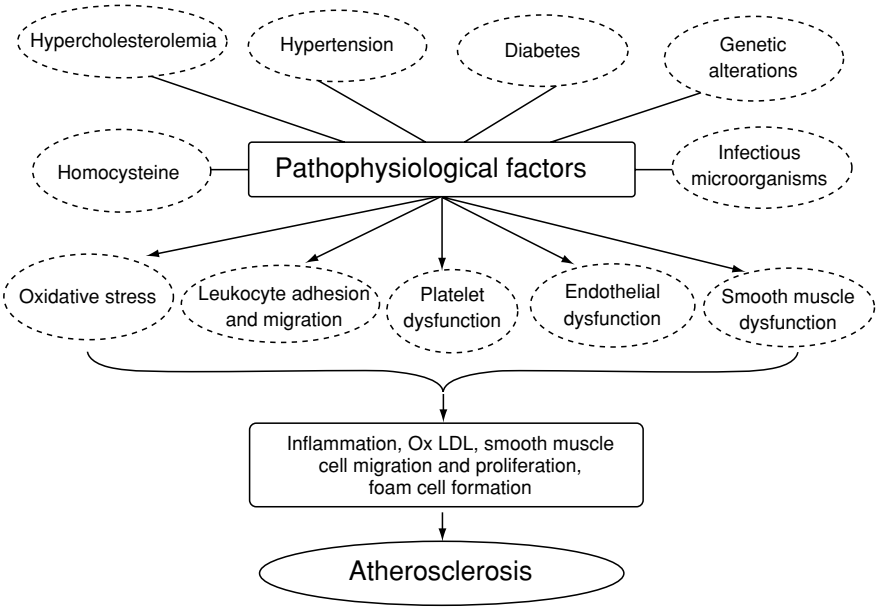

Figure 2: Pathophysiological factors that may play a role in atherosclerosis [11]

The traditional "response-to-injury" theory states that endothelial stripping absolutely caused atherosclerosis [12] however we now understand that there is a spectrum of possible insults that can contribute to the morphological changes in endothelium [13]. Endothelial cell dysfunction occurs when the normal homeostatic balance of the cell is no longer maintained, leading to either impairment in vasorelaxation or up-regulation of adhesiveness on the endothelial lining for circulating inflammatory cells [9]. Risk factors such as high amounts of low-density lipoprotein cholesterol (LDL-c), hypertension, diabetes, and cigarette smoke can lead to

endothelial dysfunction; whether it is denudation, the alteration of permeability, or the change of cytokine or growth factor secretion [13]. Regardless of the insult ensued; the common factor is the damage to the endothelium [13].

Atherosclerosis typically develops in steps that may regress or progress towards an unstable atheroma. As abovementioned, disturbance of the endothelial begins the sequence of events and the body's initial reaction is to thicken the vessel wall as a "compensatory mechanism" to the damage [13]. A common cause for inflammation in the medium or large sized arteries is the state of hypercholesterolemia (14). The arterial inflammatory response begins when excess low-density lipoproteins (LDL) infiltrate the intima layer of the artery, and through oxidation, the now oxidized LDL (oxLDL) release phospholipids that activate the endothelial cells [15,16]. The oxLDL residing in the intima layer is what activates the endothelial cells and causes an increased expression of adhesion molecules and inflammatory genes [17]. With increased expression of leukocyte adhesion molecules, cells that roll on the surface will adhere to the site of activation [18,19]. The two key adhesion molecules that are up-regulated are intercellular adhesionmolecule-1 (ICAM-1) and vascular-cell adhesion molecule-1 (VCAM-1) [8]. These adhesion molecules will facilitate attachment and transendothelial migration through the inter-endothelial junction of leukocytes, such as monocytes and lymphocytes, into the subendothelial area [8,20]. Chemokines found in the intima layer will incite the monocytes to perform such a migration [14]. Once in the subendothelial region, macrophage colony-stimulating factor (M-CSF) induces the differentiation of monocytes into macrophages [14]. With the help of scavenger receptors located on the macrophage, a broad range of particles, including the oxLDL, are "eaten up" by the macrophage, who later transform into foam cells [14,21]. In addition to scavenger receptors, toll-like receptors that also bind pathogen-like molecules, but

will as well activate the macrophage to produce inflammatory cytokines, proteases, and cytotoxic nitrogen and oxygen radical molecules [22].

In a pathological state as such, many pro-inflammatory or pro-oxidative stimuli imposed by risk factors can generate reactive oxygen species (ROS) via vascular cells [8]. The production of ROS is harmful because they can serve as second-messenger coupling molecules that transmit signals to elevate the expression of pro-atherogenic products [8]. A protection against ROS can be the antioxidant defense mechanisms. However, when the rate of ROS production exceeds the performance of the antioxidant system, the condition of oxidative stress ensues [8].

At this stage in the development, fatty streaks or xanthomas are formed due to the large lipid core. Most xanthomas can regress by means of lifestyle modifications, and will not fully develop into atherosclerotic lesions [23]. However, in the later stages of development, intimal hyperplasia occurs in which the arterial wall becomes thickened due to the migration and proliferation of vascular smooth muscle cells (VSMC) and the irregular accumulation of extracellular matrix [13]. The hyperactivity of the VSMCs will be discussed in greater detail in a later section.

With established lipid deposition and intimal thickening, the plaque can be deemed either ‘stable’ or ‘unstable’ depending on a variety of factors that may render the plaque vulnerable to rupture [24]. Many key players such as T-cells, macrophages, and mast cells [25-27] produce molecules that can negatively destabilize a lesion: inflammatory cytokines, proteases, coagulation factors, radicals, and vasoactive molecules [14]. ‘Destabilizing’ a lesion implies the destruction of collagen in the extracellular matrix, inhibiting the formation of a stable fibrous cap over the lesion, or beginning a thrombus formation [28-31]. Destabilization depends entirely on the crucial balance between collagen synthesis and collagen breakdown [8]. In one respect, VSMCs produce interstitial collagen that offers tensile strength and stability to the maintenance of the

fibrous cap [8]. However, due to the presence of T cells, macrophages are induced to produce MMPs and cysteine proteases, 2 proteases that play a key role in collagen breakdown [8, 14]. T cells may also slow down the collagen production by VSMCs by producing interferon-γ, which send signals to VSMCs to halt production [8]. The main alarming consequence of atherosclerosis is the risk that a plaque can occlude an artery, or rupture resulting in thrombus formation [13].

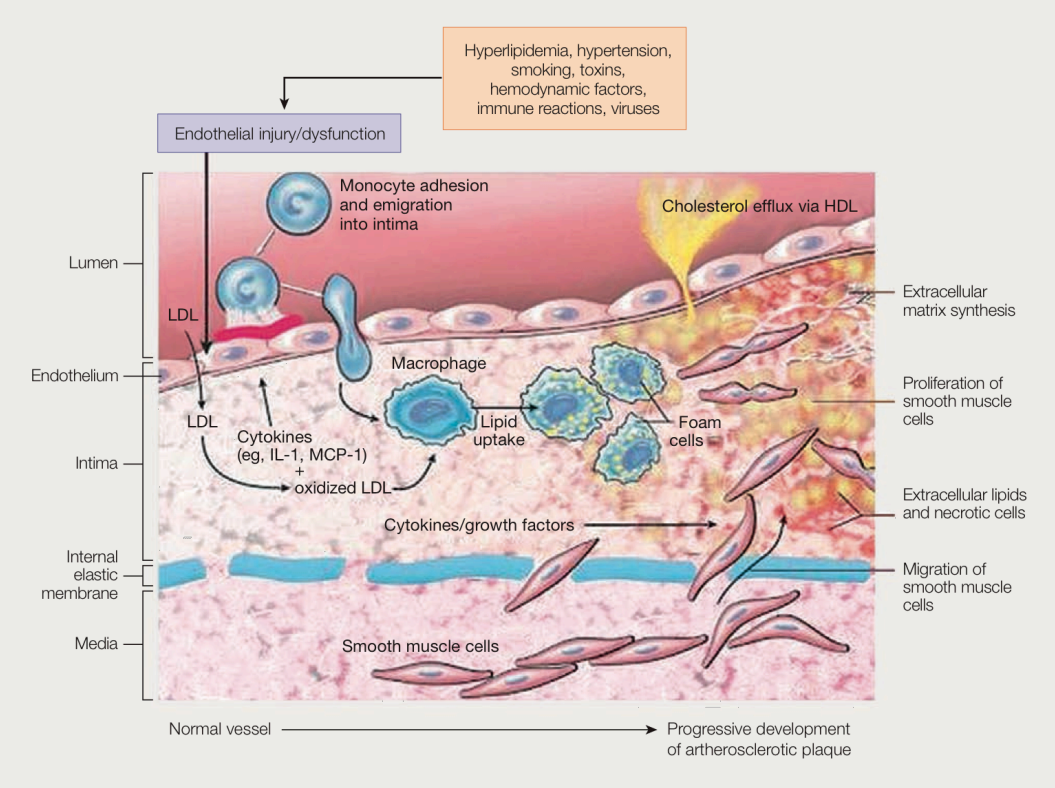

Figure 3: Schematic illustration of cellular events in atherosclerosis [32]

Pathways Leading to Atherosclerosis

It is crucial to acknowledge that atherosclerosis is not solely a 'lipid-burdened' disease. There are 3 circumstances that may lead to atherosclerosis: inflammation, autoimmunity and infection [33]. Many triggers for **inflammation** occur hand-in-hand with the risk of developing atherosclerosis such as oxidized LDL, dyslipidemia, hypertension, diabetes and obesity. These conditions may indirectly increase the risk for atherosclerosis by elevating expression of pro-inflammatory cytokines, chemokines, or adhesion molecules, all of which lead to a pro-inflammatory atherogenic pathway [33].

Atherosclerosis has also been speculated to be an **infectious** disease as some infectious agents may generate inflammatory stimuli [34]. Although infectious viruses alone may not be a predominant cause, a study showed that patients with coronary artery disease (CAD), had elevated levels of antibodies against viruses chlamydia pneumonia, helicobacter pylori, herpes simplex, or cytomegalovirus [33,35]. Extravascular infections, such as gingivitis may increase inflammatory cytokines in remote atherosclerotic lesions, while intravascular infections may provide local inflammatory stimuli [33].

Many cells of the **immune** system are involved in the pathologic processes occurring in the subendothelial region. Some of these immune cells include macrophages, T-cells, autoantibodies, autoantigens, and cytokines (interleukins, tumor-necrosis factor, interferon-γ, platelet-derived growth factor) [36]. Systemically, the inflammatory response involves acute-phase reactants serum amyloid-A, fibrinogen, and C-reactive protein [36]. Heat-shock proteins (HSPs), proteins found in most species induced by heat shock, have also been shown to promote atherogenesis [37]. Patients with early lesions, expressed higher levels of anti-HSP65 antibodies [38].

Vascular Smooth Muscle Cells & Their Involvement in Lesion Development

The VSMC is a highly specialized cell found in mature animals that expresses a unique collection of ion channels, signaling molecules, and contractile proteins to perform cell contraction [39]. These cells found in blood vessels may proliferate at a very slow rate, and express minimal synthetic activity in a healthy environment [39].

Structure and Morphology

The VSMCs essentially act as the bulk of the vessel wall, found mainly in the media layer bound together by internal and external elastic lamina [40]. In pathological conditions, VSMCs can be found in high numbers in the intimal region, in which they normally represent only a minor population. VSMCs will never reside in the adventitia layer, as it is only populated with fat cells, fibroblasts, and nerves [40]. These VSMCs have been suggested to express two main phenotypes: contractile and synthetic [40, 41]. Smooth muscle cells exhibiting a contractile phenotype are found in the media layer and are common in healthy, differentiated arteries containing many microfilament bundles [42]. These contractile cells are spindle-shaped, as seen in figure 4, and contain copious amounts of contractile fibers such as actin and myosin [40]. Upon migration to the intimal layer during atherosclerosis, the VSMCs experience a phenotype switch to the synthetic, which is typical of a diseased artery [42]. These dedifferentiated synthetic cells are more rhomboid-shaped and contain cytoplasm that have a prevalence of rough endoplasmic reticulum and matured Golgi apparatus [42].

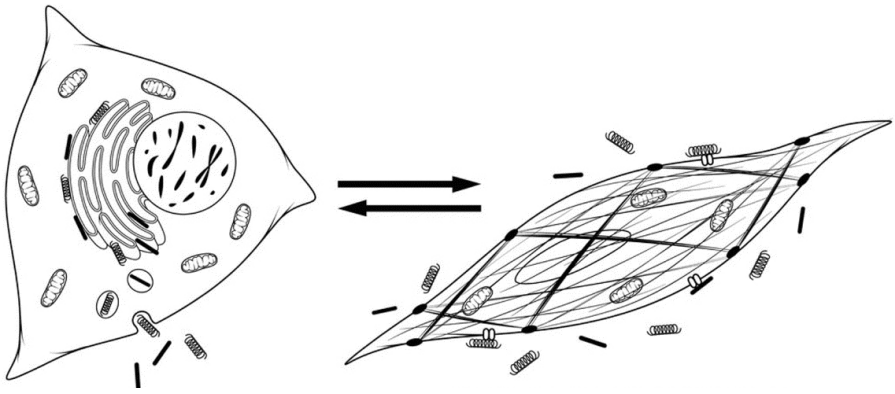

Figure 4: Phenotype plasticity of the VSMCs. The cell on the right represents a contractile, differentiated VSMC, while the cell on the left represents a dedifferentiated synthetic cell responding to vascular injury [43].

VSMCs: Function and Roles

VSMCs share two primary functions in the arterial wall: contractility and structure [40]. In arteries and veins, the main structural components are the VSMCs and the extracellular matrix, composed of collagen and elastin [13,40]. VSMCs maintain a contractile role due to the large expression of proteins smooth muscle (SM) α-actin isoform, myosin heavy chain (MHC), smoothelin, alpha tropomyosin, calponin, caldesmon, myosin regulatory light chain 2, intermediary filaments vimentin and desmin, as well as many other proteins [40,44,45]. With the support of the array of proteins, the cells can maintain tone and play a major part in vasoconstriction following physiological or pharmacological stimuli [13]. In other words, following a pulse wave, the layer of VSMCs can act as an elastic reservoir for energy because it in turn regulates the blood flow following nervous or humoural stimulation [40]. The phenotype of the cells in the media layer is best described as "contractile", however as we will discuss in the next section, this phenotype is altered once intimal hyperplasia occurs [46]. VSMCs are unique in that they are not terminally differentiated, which is classic of cardiac or skeletal muscle. They can experience plasticity that allows phenotype adaptations in response to cellular environmental stimuli [47].

The Diseased Intima: Phenotype Alteration, Migration & Proliferation

The proliferation of smooth muscle cells is a response-to-injury and a necessary process to occur at the onset of inflammation [48]. There are three main phases in the response that cause movement of VSMCs and intimal hyperplasia. During the initial onset, replication of VSMCs occurs, typically stimulated by the release of basic fibroblast growth factor (bFGF) from the dead and damaged cells [40]. Once having multiplied, the smooth muscle cells will migrate towards the intima from the internal elastic lamina of the medial layer. During the migration of the VSMCs, a change of phenotype occurs from contractile to fibroblast-like synthetic phenotype [47]. Many molecules mediate this travelling action; however platelet-derived growth factor (PDGF) plays a crucial role and has been debated to be mitogenic for VSMCs [40]. Once having reached the intimal region, the third phase entails proliferation of the cells and secretion of extracellular matrix with the ability to form a stable "fibrous cap" between the lipid lesion and arterial lumen [49].

The proliferatory phase for the VSMCs will occur under the influence of an assortment of growth factors and cytokines, and only upon completion, will the extracellular matrix be released and accumulated in abnormally large amounts [23]. Factors that can act as chemotactic agents for the smooth muscle cells are; PDGF, acidic fibroblast growth factor (aFGF), bFGF, α-thrombin, and insulin-like growth factor 1 (IGF-1) [13,50-53]. These compounds are termed 'chemotactic' because they will affect the normal activity of the VSMCs in the media and favor changes towards migration and proliferation of the cells [13]. In contrast, there are substances produced that inhibit VSMC proliferation and intimal growth in response to the vascular stimuli: heparin, TGF-β, and nitric oxide (NO) [54]. In effect, these chemotactic growth factors along with

infiltrating leukocytes and adhesion molecules allow for a positive feedback loop that continue the vicious cycle of VSMC hyperactivity and increased intimal thickening [13,55].

Aside from the increased VSMC activity towards the intima, the extracellular matrix plays a perilous role in plaque development. The synthetic VSMCs in the intima are different from the contractile VSMCs in that they secrete proteins of the extracellular matrix, such as collagen I and II [23]. The components of the extracellular matrix offer tensile strength for the arterial wall; due to the VSMC's production of collagen fibers (types I, III, V), small proteoglycans, and the elastic membranes [56,23]. In an atherosclerotic plaque, there is exaggerated matrix deposition that severely narrows the vessel lumen [23]. Moreover, the distribution of collagen and elastin is distinct, since the intimal extracellular matrix exhibits much more collagen and much less elastin than does the medial extracellular matrix [23]. It has recently been speculated that perhaps macrophages may also be able to produce some components of the extracellular matrix by means of secretion of TGF- β [23]. With excessive extracellular matrix, the fibrous cap atheroma will begin to form. The atheroma entails the accumulation of partially necrotic foam cells from the earlier inflammatory phase, which is separated from the vessel lumen by VSMC-derived fibrotic tissue [23].

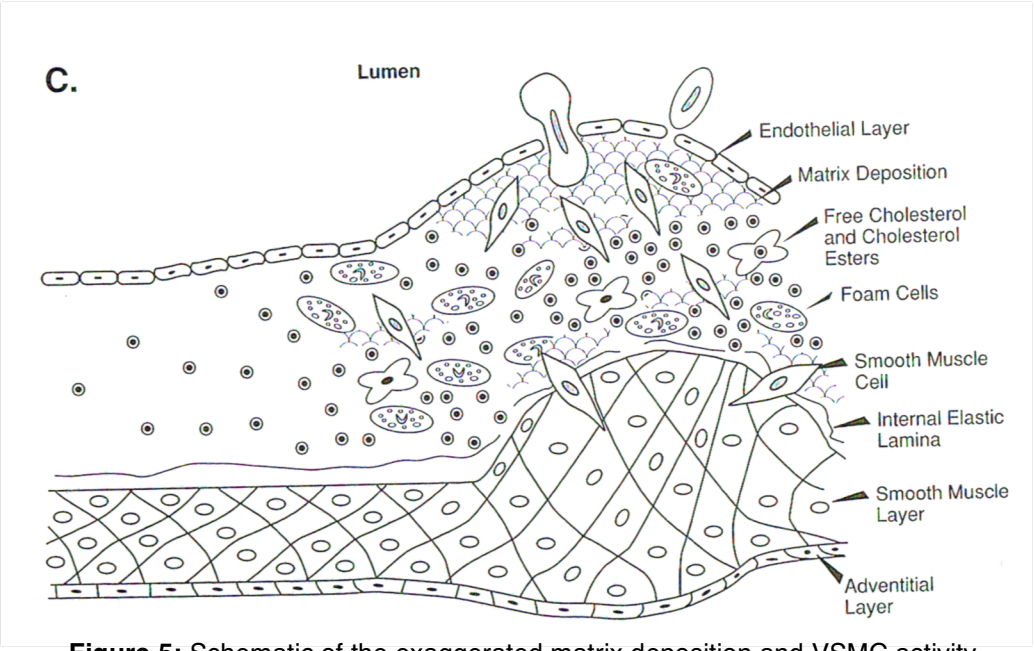

Figure 5: Schematic of the exaggerated matrix deposition and VSMC activity, resulting in plaque development [13].

Low-Carbohydrate Diets and Cellular Metabolism

Under normal homeostatic conditions, protein provides a small amount of 2-5% of the body's energy requirements; predominant contributions for energy originate from carbohydrates (CHO) and fats [57]. In situations where CHO availability is limited, the body must compensate by utilizing its stores of glycogen in the liver and muscle to maintain normal blood glucose levels and supply the cells with glucose [58]. In diabetic scenarios where glucose is not taken up into the cells, or states of glycogen depletion, which usually begin at 48 hours of total CHO restriction, cellular fuel adaptations occur and a shift towards the use of fatty acids transpires; fat mobilization > fat oxidation [57,58]. Fat oxidation begins to dominate once glycogen stores are exhausted and gluconeogenesis does not suffice [58]. With the same energy sources being using

but in different proportions, the fuel source shifts from being “glucocentric” to “adipocentric” [59].

When triglycerides are forced to be broken into fatty acids and glycerol for energy use, a process called β-oxidation occurs in the mitochondria to convert fatty acids into a readily useful form [60]. The fatty acids are converted into acetyl-CoA and can enter the Krebs Cycle in the same fashion as glucose. In conditions lacking CHO and fat for energy, protein catabolism offers an important source of energy in a few ways. Before any amino acids (AA) can be oxidized, removal of the amino group must occur. In one instance, the detached amino group can get transferred and given to another AA (ketoacid), to form a new AA [57]. This process is called *transamination.* Otherwise, oxidative deamination can occur when the amino group may be removed and become free ammonia (NH_3) [61]. Nevertheless, when the amino group is removed, a carbon skeleton is remaining and may become oxidized or converted to form different substances capable of entering the Krebs Cycle: CO_2, H_2O, acetyl-CoA, oxaloacetate, α-ketoglutarate [61].

Project Overview

Dieting trends and use of supplementation is practiced widely in North America for various reason, many of which focus on weight loss or disease prevention. The goal of this thesis is to better understand how manipulating macronutrient distribution in the diet can have profound effects on a cellular level both beneficial and harmful. Animal source protein, such as casein or whey is commonly used for protein supplementation with the goal of gaining muscle mass. Casein protein is frequently used for custom-made animal diets for research purposes. Animal proteins are of interest to researchers due to their potential hypercholesterolemic effect in humans and animals. This matter will be investigated in depth in the published review paper to follow.

Another common use of dieting is a low-carbohydrate high-protein diet, generally referred to as the "Atkin's diet". This carbohydrate-eliminating method of weight loss has yet to be studied in regards to cardiovascular health, more specifically vascular smooth muscle cells in atherosclerosis and mitochondria functioning in congestive heart failure. Reduction of carbohydrates has a direct implication in the normal cellular metabolism, whereby a shift from a carbohydrate energy source to a fat energy source occurs. These projects aim to assess whether this shift can affect the vascular smooth muscle cells and their role in development in atherosclerosis in the thoracic aorta, more specifically their phenotype shift from contractile to synthetic phenotype. As well, the mitochondrial respiration will be assessed in the cardiac tissue whether they function better in a lipid-rich environment. Information from this thesis can provide ample information to health care professionals and individuals engaging in dieting practices, to better understand the risks and safety.

II

The role of casein in the development of hypercholesterolemia

Olivia Hanna Koury, Celena Scheede-Bergdahl, Andreas Bergdahl

Contribution of Authors

Olivia Hanna Koury: preparation of manuscript.

Celena Scheede-Bergdahl: editing of manuscript.

Andreas Bergdahl: preparation of manuscript.

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MINI REVIEW

The role of casein in the development of hypercholesterolemia

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Abstract Atherosclerosis remains the leading cause of severe cardiovascular complications such as cardio- and cerebrovascular events. Given that prevention and early intervention play important roles in the reduction of cardiovascular complications associated with atherosclerosis, it is critical to better understand how to target the modifiable risk factors, such as diet, in order to best minimize their contributions to the development of the disease. Studies have shown that various dietary sources of protein can affect blood lipid levels, a modifiable risk factor for atherosclerosis, either positively or negatively. This clearly highlights that not all proteins are "created equal." For example, consumption of diets high in either animal- or vegetable-based sources of protein have resulted in varied and inconsistent effects on blood cholesterol levels, often depending on the amino acid composition of the protein and the species investigated. Careful consideration of the source of dietary protein may play an important role in the prevention of atherosclerosis and subsequent cardiovascular complications. Given the recent focus on high protein diets, an emphasis on controlled studies in the area is warranted. The goal of this review is to present the current state of the literature that examines the effects of casein, a commonly utilized animal-based protein, on blood cholesterol levels and the varying effects noted in both animals and humans.

Keywords Casein protein · Hypercholesterolemia · Soy protein · Lipoprotein · Cardiovascular disease

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Cholesterol and the development of atherosclerosis

Atherosclerosis lies at the root of many serious cardiovascular complications such as myocardial infarction, stroke, gangrene, intermittent claudication, and limb amputation [39]. The initiation of the atherosclerotic process depends mainly on the state and function of the endothelial layer, which represents the demarcation between the vessel wall and the blood [15]. Endothelial dysfunction is characterized by two aspects: a reduction of the bioavailability of nitric oxide, which leads to impaired vasoreactivity, and the activation of the endothelial cells [7, 51]. Taken together, these features induce a pro-inflammatory, proliferative, and pro-coagulatory state, all of which contribute to the progression of atherogenesis [1]. Factors associated with endothelial dysfunction include smoking, oxidative stress, diabetes, metabolic dysfunction, obesity, hypercholesterolemia, and hypertension [20] (Fig. 1).

High-density lipoproteins (HDL) are considered to be a negative risk factor for the development of cardiovascular disease and have been casually referred to as "good" cholesterol. The cardioprotective effects of HDL

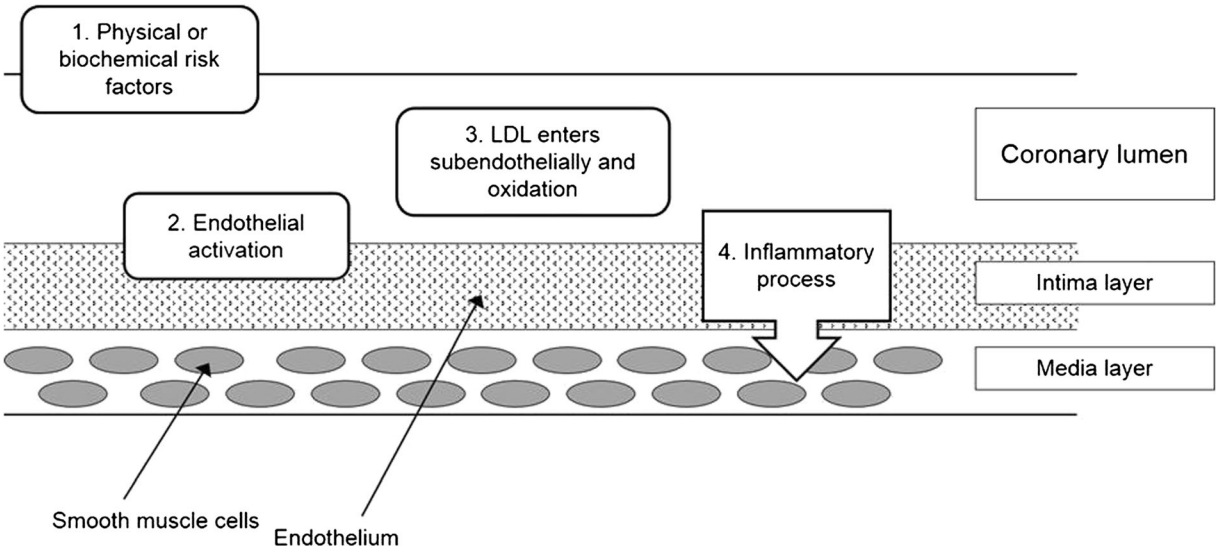

Fig. 1 Schematic view of the arterial wall and the steps in atheroma formation. Risk factors such as a biochemical imbalance (high LDL) trigger an endothelial activation beyond the normal noxious stimuli. LDL enters the subendothelial layer and become oxidized to allow attraction of monocytes, cytokines, and other inflammatory cells to support the inflammatory process

are due to its role in reverse cholesterol transport (RCT), a key process that regulates cholesterol clearance from the systemic circulation. The purpose of RCT is the removal of excess (free) cholesterol from peripheral cells and reuptake by the liver for eventual bile salt synthesis and excretion [8]. In the early stages of atherosclerosis, a higher than normal concentration of circulating low-density lipoproteins (LDL) results in their penetration into the subendothelial layer of the blood vessel. LDL, transported by apolipoprotein B (ApoB) into the vascular wall, becomes biochemically modified and, subsequently, triggers an inflammatory response [21]. There are three histological features of the unstable atheroma: (1) the large lipid core which is present when the abovementioned RCT is incapable of regulating blood cholesterol levels, (2) the abundance of inflammatory cells, and (3) a thin fibrous cap [17, 22]. The atheroma is not only a collection of cholesterol, waste, and fibrotic tissue but is also a lesion composed of endothelial and smooth muscle cells with infiltrating leukocytes and other inflammatory cells [32]. Essentially, the initial endothelial dysfunction leads to the fatty streak formation and, ultimately, fibrous cap formation [39].

The risk of developing atherosclerosis and consequent ischemic heart disease increases with the presence of pro-atherogenic substances such as intermediate density lipoproteins (IDL), low-density lipoproteins (LDL), and very low-density lipoproteins (VLDL) [33]. Evidence in the literature has suggested that certain proteins appear to exert a greater effect on blood cholesterol levels than others [50]. Given that the recent trend towards high protein diets in the pursuit of weight loss and reduction of chronic disease risk, it remains imperative to fully appreciate the associations between certain protein types and the potential for increased cardiovascular disease risk. This increased risk may occur despite successful weight loss. The goal of this review paper is to examine whether casein, a dietary source of protein, has an effect on blood cholesterol and whether it can be considered a positive risk factor for the development of atherosclerosis.

What is casein and does it have a role in the atherosclerotic process?

Over the last five decades, there has been a steady interest in the abilities of certain proteins to promote either a pro- or anti-atherogenic effect. In particular, casein has often been included in studies as an animal source of protein [50]. Milk products contain two main protein components: whey and casein. Whey protein represents approximately 18 to 20 % of mammalian milk protein, while casein represents the remaining 80–82 % [4]. Casein is regarded as one of the most

nutritive milk proteins, as it contains all common amino acids and is rich in essential amino acids (EAA) [45]. Purified casein is produced from skim milk by a processing technique where the protein is separated from the whey, dried, and then resolubilized [9]. Isolated milk casein forms micelle complexes when dispersed in the water phase of milk. The micelle structures have five different subunits of the casein subtype: α-casein, α-2 casein, β-casein, κ-casein, and γ-casein. Common among these five structures are the calcium-phosphate bonds that hold them together and that they all contain salt and water [9].

The primary difference between casein, whey protein, and other high-quality proteins is the rate of digestibility. Casein is considered to be a *slow*-digesting protein because it curdles or gels in the stomach, thus delaying release in the intestines. This results in a gradual but steady rise in blood amino acid concentration following ingestion [9]. Since the blood amino acid concentrations are kept relatively low, it slows but extends the rate of protein synthesis [16]. Casein also demonstrates anti-catabolic properties, which simultaneously inhibits protein breakdown [4, 6]. In situations where weight loss is desired, the anti-catabolic properties of casein result in it being the preferred source of protein for hypocaloric diets [5]. In light of these characteristics, casein is attractive for many weight loss programs that include high protein intake.

As early as the 1970s and 1980s, animal studies reported that casein increased serum cholesterol levels, thus playing a role in the development of atherosclerosis. Previously, it was believed that cardiovascular disease and atherosclerosis were a result of the amount of fat in the diet and, in particular, the cholesterol and saturated fats. In light of results seen in animal studies, a high casein diet may also be considered a risk factor in the development of atherosclerotic plaque. Casein-mediated hypercholesterolemia has been shown to develop independently of exogenous cholesterol and saturated fat consumption [28]. Studies have been performed in order to compare lipid profiles upon adherence to diets that vary in amount of cholesterol, cholesterol-free semi-purified diets, and various protein sources. Early studies have shown that soy protein, a source of vegetable protein, appears to play a protective role in the vasculature and reduces the concentration of total cholesterol and LDL, contrary to the detrimental effects of casein [10]. A negative association between soy protein intake and the development of coronary heart disease and nonfatal myocardial infarctions was also shown in a group of middle-aged and older Chinese women [52], although these protective effects of soy are not detected in all studies [24]. The association between animal-based protein and cardiovascular disease is also supported by a meta-analysis of five prospective studies that compared mortality in vegetarians and non-vegetarians. This analysis showed that subjects who consumed animal protein had a 24 % higher mortality from ischemic heart disease, even after controlling for potential confounding factors such as age, sex, smoking status, alcohol, habitual exercise, education, and body mass index [29]. Given the potential detrimental effects of casein and other animal-based proteins in the development of vascular disease, as suggested in studies such as these, it is important to consider dietary protein source when recommending high protein diets rather than considering all proteins equal.

The effects of casein in the animal model

Research conducted in male New Zealand white rabbits by Huff and colleagues clearly demonstrated that changes in body weight, plasma cholesterol, triglyceride levels, as well as liver cholesterol occurred upon varying the dietary protein source (i.e., either animal or plant protein) [26]. The diet used in the Huff study was termed a "low-fat semi-purified diet", consisting of either 27 % casein or soy isolate, along with 60 % dextrose, 5 % celluflour, 4 % salt mix, 3 % molasses, and 1 % corn oil. The 16 animals were divided into 2 groups: 8 received a diet rich in casein and 8 received a diet rich in soy protein isolate. This low-fat semi-purified diet was given to both groups for 10 months, resulting in significantly lower levels of mean plasma cholesterol in the soy isolate group as compared to the casein semi-purified group. After 10 months, the casein-fed animals ended up with higher mean triglyceride levels, higher liver cholesterol (Table 1), and developed atherosclerotic lesions, particularly in the aortic arch region. Despite the negative effects on plasma lipids and cholesterol, casein-fed animals gained less weight than the soy-fed animals (2.9 and 3.3 kg, respectively).

Plasma VLDL, LDL, and IDL levels were all significantly higher in the casein-fed versus the soy-fed animals (Table 2). Huff and colleagues concluded that this hypercholesterolemic state must be the result of cholesterol that is endogenously produced by the liver or

Table 1 The condition of the rabbits following the observance of a casein- or soy-rich diet. Cholesterol and triglyceride contents in the plasma and liver were all significantly elevated, excluding the liver triglyceride (adapted from [26] with permission)

	Casein (mg/dl)	Soy protein (mg/dl)
Mean plasma cholesterol[a] (mg/dl)	247±12	66±3[b]
Liver cholesterol (mg/g wet wt)	6.6±1	3.3±0.2[b]
Mean plasma triglyceride[a] (mg/dl)	95±4	58±3[b]
Liver triglyceride (mg/g wet wt)	7.0±0.7	6.6±0.3

Lipid profile in rabbits fed either casein or soy protein diet for 10 months. Results are expressed as a mean ± SE for 8 rabbits in each dietary group

[a] The overall mean ± SE for the entire 10-month period

[b] Significantly different from the casein-fed group $P<0.01$ from Student's t test

intestine in response to the manipulation of dietary protein source [26]. This significant rise in IDL, the main transporter of cholesterol, has been observed under similar conditions since although the underlying cause remains speculative [44].

In the Huff study, it is interesting that both the casein and soy diets lacked exogenous cholesterol and were low in saturated fats. From these, it is evident that the source of protein directly affects the distribution pattern and concentration of cholesterol being transported in the blood [26]. The amino acid composition of the protein is as important as the protein source [11]. It is known that amino acids vary in their effects on serum cholesterol concentrations, as well as further variations when ingested as proteins [11]. Illustrating this concept, the amino acids lysine and methionine contribute to the development of hypercholesterolemia, whereas arginine is able to counteract this effect [30, 31]. The essential amino acids found in casein have been hypothesized to be responsible for the observed increase of total cholesterol and LDL [11]. By replacing casein with isolated soy protein, which has a different amino acid composition, the increases in total and LDL cholesterol content in the serum associated with casein can be avoided [3].

Table 2 The varying distribution of plasma cholesterol that developed among the four lipoprotein classes: VLDL, IDL, LDL, and HDL, as well as the total plasma concentration. Differences with both diet groups in all lipoprotein classes are significant, excluding the HDL (adapted from [26] with permission)

Density class	Casein (mg/dl)	Soy protein (mg/dl)
VLDL d<1.006	76±6	11±3[a]
IDL 1.006<d>1.019	132±14	25±6[a]
LDL 1.019<d>1.063	46±6	10±2[a]
HDL 1.063<d>1.21	19±4	12±3
Total plasma concentration	275±25	58±6[a]

Plasma cholesterol distribution among lipoprotein classes. Results are expressed as mean ± SE for 6 rabbits in each dietary group

[a] Significantly different from the casein-fed rabbits ($P<0.01$) from Student's t test

Terpstra and colleagues reported similar results, but their data also demonstrated the dose effect of casein fed to Zucker strain rats [46]. Their study included six groups of animals fed with either a commercial diet with no cholesterol, a commercial diet with 1.2 % cholesterol, or four types of semi-purified cholesterol enriched diets (20 % casein, 50 % casein, 20 % soybean, or 50 % soybean (g/100 g of feed)). The results revealed a more prominent hypercholesterolemic effect occurring in diets with a higher percentage of casein. The same effect was observed in rabbits [25, 46], as well as in pigeons [34, 35].

Evidence in the literature supports the notion that the casein-mediated cholesterol increases may be biphasic in nature: extremely low and extremely high amounts of casein in the diet appear to have the greatest impact on blood cholesterol levels [27, 40]. Increases in cholesterol were most apparent with either diets containing relatively small amounts (5 %) or large amounts (40 to 60 %) of casein. Diets consisting of moderate amounts of casein (i.e., 20 %) appeared to produce the smallest effects [19]. Gender also appears to play a role: Female rats were more predisposed to developing hypercholesterolemia in response to casein ingestion [47]. This observation had also been previously reported by Filios and colleagues [19] with blood cholesterol levels doubling in magnitude in female versus male rats.

Research conducted by Hermus and colleagues focused on demonstrating the different combinations of protein with gelatin on serum cholesterol levels and body weight gain in rabbits [23]. The team set up four experimental groups: (1) semi-purified diet containing strictly casein as the protein source; (2) casein and gelatin; (3) casein, gelatin, and fish protein; and (4) casein, gelatin, fish protein, and soy protein. After 58 weeks of diet adherence, the group fed the diet consisting of casein only demonstrated a growth-retarding effect compared to the other groups who achieved normal growth. Additionally, the casein group

also achieved a hypercholesterolemic state that was unseen in the other three experimental groups. This study demonstrated that the addition of alternate protein sources to the diet was able to blunt the hypercholesterolemic effects of casein.

Overall, it is understood that the atherosclerotic process can be initiated by hypercholesterolemia, with serum total cholesterol and lipoproteins being well-established risk factors for the disease [21]. Although elevated concentrations of serum cholesterol contribute to the development of fatty streaks, this is not the sole reason for disease susceptibility [28]. Other known risk factors, such as family history, obesity, chronic hyperglycemia, and physical and/or biochemical injuries, have also been implicated [21, 28]. To fully appreciate the origins and mechanisms involved in the progression of atherosclerosis, what has been learned from studies involving possible dietary sources of cholesterol must also be considered. When certain animals are fed with a casein-rich diet, there is a higher correlation with lipophilic plaques and high serum cholesterol content than a diet consisting of plant protein [28]. The lipoprotein density concentration is also altered, depending on whether a plant or animal protein source is considered [37]. Theories suggest that soybean protein contain saponins, which are protective against hypercholesterolemia [42]. It is also thought that dietary fiber increases absorption of bile acids in the intestine. This will result in loss of bile acid through fecal excretion which will be compensated by stimulation of hepatic conversion of cholesterol into bile acids [41]. The main question is how *casein* protein causes an elevation in cholesterol, such that the protein source is as detrimental as the source of fat [26]. What provokes this mechanism to increase cholesterol to such a level as to induce atherogenic plaque?

Many speculations and theories have been put forth over the past 30 years as to how and why casein ingestion raises cholesterol levels and why certain species are more susceptible to the effects of casein. The activity and concentration of enzyme alkaline phosphatase play an important role since it has the potential to dephosphorylate casein and prevent accumulation of phosphopeptides [36]. In 1988, Van Der Meer and colleagues conducted studies that involved feeding both rabbits and rats a similar diet that included elevated casein content in order to investigate intestinal absorption and bile acid excretion. They reported that casein induces a hypercholesterolemic effect in rabbits due to low intestinal phosphatase activity and with a high glycine conjugation of bile acids, whereas in the rat, where little effect of casein was noted, the conjugation of bile acids occurs primarily via other amino acids, such as taurine [48].

Another potential factor that may contribute to the effects of casein is the LDL receptors. It has been reported that animals fed casein-enriched diet had a downregulation of hepatic LDL receptors preceded by an increase in plasma cholesterol [12]. Other studies have also shown that casein stimulates LDL ApoB synthesis, therefore increasing the circulating LDL [11].

What effect does casein have in a human model?

Contrary to the observations seen in animals, the majority of intervention studies that have investigated the effects of plant and animal protein on serum cholesterol levels in humans have reported inconsistent effects. In 1983, Sacks and colleagues tested whether dairy protein (casein) or soy protein would have an effect on plasma cholesterol in 13 strict vegetarians. The study design consisted of a 1-week pre-intervention period, during which, baseline measurements such as body weight, cholesterol profile, triglycerides, and VLDL-c/TG ratio were measured. Following the baseline period, all 13 subjects were split into groups that followed two phases: a diet enriched in casein for 20 days and then soy for 20 days or vice versa. Results yielded no significant changes in LDL or protective HDL cholesterol from baseline. As well, there was no difference of lipid profile or lipoproteins in the soy and casein groups during their 40-day intervention [43].

Another study conducted by Van Raaij and colleagues looked at similar aspects, but in healthy non-vegetarian subjects eating a "western" simulated diet. All 69 participants began by eating a casein-soy or "cassoy" diet for a control period of 10 days in order to establish baseline measurements for the remainder of the experimental design. In this cassoy diet, 65 % of the protein content was a 2:1 mixture of casein and soy, respectively. Following the 10-day control period, individuals were divided into three groups for 28 days: maintenance cassoy diet, casein diet, or soy diet. Interestingly, there were barely any changes seen between the experimental casein and soy groups in regard to total cholesterol. Subjects adhering to the casein-enriched diet did not demonstrate any significant changes in

lipoprotein fractions. On the other hand, the soy group demonstrated improvements in the LDL and HDL concentrations as compared to the casein group, and the reductions in LDL were also significant within the soy group from their baseline measurements [49]. The improvements in LDL-c and HDL-c were also seen in another study with participants eating a cholesterol-enriched casein or soy diet [38]. VLDL concentrations remained unchanged in both study groups.

Crouse and colleagues conducted a similar study with healthy individuals having elevated LDL concentrations between 3.62 and 5.17 mmol/L. Subjects maintained a casein-rich diet or a soy-rich diet with varying amounts of isoflavones for 9 weeks. Following this dietary intervention, LDL-c and total cholesterol lowered significantly from baseline in individuals in the soy group, with a dose-dependent relationship between cholesterol improvements and isoflavone amount. There was no significant improvement in HDL-c or TG concentration between the groups [14], suggesting that casein supplementation is not an effective intervention for individuals with pre-existing high cholesterol.

From these studies performed on humans, the results suggest that casein protein may not have such a pronounced effect on cholesterol levels as seen in certain animal models, such as rabbits. The studies share mixed results and conclusions. A soy diet would be more beneficial for lowering cholesterol levels in hypercholesterolemic patients since casein-enriched diets demonstrate few advantages in lowering LDL-c or TG. In healthy subjects, on the other hand, it seems as though casein and soy diets lack influence on cholesterol levels [43]. However, it should be pointed out that, in order to better mimic the study design in animals, the human experiments would need to be conducted for longer periods of time and/or administered at earlier periods in their lives [49]. The important point here is to consider whether the lack of effect is indeed a phenomenon in the human model or whether it is due to limitations in study design. Further work is needed in order to objectively determine the safety of diets high in casein.

Clinical implications of a high casein diet

Meal replacements (MR) containing elevated amounts of plant or animal protein have proven successful in promoting weight loss in obese patients. In a randomized controlled trial by Anderson and colleagues [2], patients consuming a MR with high casein for 16 weeks had a tendency towards more weight loss and greater fat loss than individuals on high soy diets. Although associated with only modest weight loss, the individuals consuming soy MR showed promising improvements in their cardiovascular risk profile (i.e., LDL, TC, visceral fat, and systolic blood pressure). The period following weight loss is crucial to successfully sustain weight reduction, and for this, high casein diets have been found to be useful [13].

Attention must also be drawn to the popular use of protein supplementation following exercise training. Postprandial protein synthesis has been studied, and the data indicates that amino acids dictate future protein synthesis, breakdown, and oxidation [6]. Protein shakes of whey and casein are frequently used to maintain and increase muscle mass, hence protein synthesis or anabolism following exercise. As compared to whey protein, ingestion of casein protein in a meal or drink may only induce a small increase in protein synthesis, but is associated with a substantial decrease in protein breakdown [6]. These physiological processes are possible due to the retarded increase in plasma AA concentrations. Without the pronounced hyperaminoacidemic peak associated with whey, casein offers a "longer lasting" rise in AA levels and sustains protein breakdown inhibition. Casein supplementation, compared to whey, has been shown to improve body composition resulting in decreased percent body fat; an increase of lean mass; and greater muscle strength in legs, chest, and shoulders [18]. These effects are most likely due to casein's anticatabolic property. Depending on individual aims or motives, athletes may opt for casein as their choice of protein supplementation for the aforementioned reasons.

Conclusion

Casein is not only naturally present in foods containing dairy such as milks and cheeses but is also extensively utilized in its purified form as a powdered protein supplement. For decades, saturated fats, and cholesterol have been deemed responsible for the development of cardiovascular disease and conditions such as atherosclerosis and the "clogging of arteries." Studies have also found that casein protein is just as aversive as fats for some animal species. Casein can have a negative

impact on the serum cholesterol concentration and raise it to levels that pose a severe danger to the lipid profile. There have been speculations that casein is responsible for the disruption of bile acid binding in the small intestine, leaving elevated levels of free bile acids to be re-absorbed. It is key to clearly understand whether hypercholesterolemia is enough to induce atherosclerosis and simulate endothelial dysfunction and to what extent does it raise cholesterol and cause atherosclerosis. Another important aspect that would require further investigation is the possibility that humans may manifest the same hypercholesterolemic dangers of casein if given for longer periods of time comparable to animal studies. Although healthy humans are assumed to be less sensitive to dietary modifications than animals, there may be changes noted if the human studies would parallel animal studies in terms of time periods and administration [49]. Would a diet low in fats but high in animal protein be harmful to individuals and pose cardiovascular risks? The potential dangers of casein should serve as a red flag for dieters, trainers, physicians, or nutritionists alike, especially when considered for populations who may already be considered at risk for cardiovascular complications. In regard to supplementation, patients in a rehabilitation setting due to conditions such as cancer cachexia or sarcopenia may also be administered large doses of soy, whey, or casein. Also of concern are people who exercise train and consume exceptionally high concentrations of whey or casein protein supplementation in order to increase protein synthesis and muscle mass. Understanding the implications of a high casein protein diet is vital in order to assess the health status and long-term lipid profile of an individual adhering to such a diet.

References

1. Anderson TJ (1999) Assessment and treatment of endothelial dysfunction in coronary artery disease and implications for therapy. J Am Coll Cardiol 34:631–638
2. Anderson JW, Fuller J, Patterson K, Blair R, Tabor A (2006) Soy compared to casein meal replacement shakes with high energy-restricted diets for obese women: randomized controlled trial. Metabolism 56:280–288
3. Anthony MS, Clarkson TB, Bullock BC, Wagner JD (1997) Soy protein versus soy phytoestrogens in the prevention of diet-induced coronary artery atherosclerosis of male cynomolgus monkeys. Arterioscler Thromb Vasc Biol 17:2524–2531
4. Antonio J, Incledon T (2001) The anticatabolics. In: Antonio J, Stout J (eds) Sports supplements. Lipincott Williams & Wilkins, Philadelphia, pp 111–136
5. Bendtsen LQ, Lorenzen JK, Bendsen NT, Rasmussen C, Astrup A (2013) Effect of dairy proteins on appetite, energy expenditure, body weight, and composition: a review of the evidence from controlled clinical trials. Adv Nutr 4(4):418–438
6. Boirie YM, Dangin P, Gachon P, Vasson MP, Maubois JL, Beaufrere B (1997) Slow and fast dietary proteins differently modulate postprandial protein accretion. Proc Natl Acad Sci U S A 94(26):14930–14935
7. Bonetti PO, Lerman LO, Lerman A (2003) Endothelial dysfunction—a marker for atherosclerotic risk. Arterioscler Thromb Vasc Biol 23:168–175
8. Burges JW, Sinclair PA, Chretien CM, Boucher J, Sparks DL (2006) Reverse cholesterol transport. In: Cheema SK (ed) Biochemistry of atherosclerosis. Springer, New York, pp 3–22
9. Campbell B (2012) Dietary protein efficiency: dietary protein types. In: Lowery LM, Antonio J (eds) Dietary protein and resistance exercise. CRC, Boca Raton, pp 95–114
10. Carroll KK (1991) Review of clinical studies on cholesterol-lowering response to soy protein. J Am Diet Assoc 91(7):820–827
11. Carroll KK, Kurowska EM (1995) Soy consumption and cholesterol reduction: review of animal and human studies. J Nutr 125:594S–597S
12. Chao YS, Yasmin TT, Alberts AW (1982) Effects of cholestyramine on low density lipoprotein binding sites on liver membranes from rabbits with endogenous hypercholesterolemia induced by a wheat starch-casein diet. J Biol Chem 257:3623–3627
13. Claessens M, Van Baak MA, Monsheimer S, Saris WHM (2009) The effect of a low-fat, high-protein or high-carbohydrate ad libitum diet on weight loss maintenance and metabolic risk factors. Int J Obes 33:296–304
14. Crouse JR, Morgan T, Terry JG, Ellis J, Vitolins M, Burke GL (1999) A randomized trial comparing the effect of casein with that of soy protein containing varying amounts of isoflavones on plasma concentrations of lipids and lipoproteins. Arch Intern Med 159:2070–2076
15. Cullen P, Rauterberg J, Lorkowski S (2005) The pathogenesis of atherosclerosis. In: Von Eckardstein A (ed) Atherosclerosis: diet and drugs. Springer, New York, pp 3–70
16. Dangin M, Guillet C, Garcia-Rodenas C, Gachon P, Bouteloup-Demange C, Reiffers-Magnani K, Fauquant J, Ballevre O (2003) The rate of protein digestion affects protein gain differently during aging in humans. J Physiol 549(2):635–644
17. Davies MJ (1997) The composition of coronary-artery plaques. N Engl J Med 336(18):1312–1314
18. Demling RH, DeSanti L (2000) Effects of a hypocaloric diet, increased protein intake and resistance training on lean mass gains and fat mass loss in overweight police officers. Ann Nutr Metab 44(1):21–29
19. Filios LC, Naito C, Andrus SB, Portman OW, Martin RS (1958) Variations in cardiovascular sudanophilia with changes in the dietary level of protein. Am J Physiol 194:275–279
20. Forrester JS (2008) The pathogenesis of atherosclerosis and plaque instability. In: Holtzman JL (ed) Atherosclerosis and oxidant stress. Springer, New York, pp 1–10

21. Gross MD (2008) Lipids, oxidation, and cardiovascular disease. In: Holtzman JL (ed) Atherosclerosis and oxidant stress. Springer, New York, pp 79–96
22. Hansson GK (2005) Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 352(16):1685–1695
23. Hermus RJJ, West CJ, Van Weerden EJ (1983) Failure of dietary-casein-induced acidosis to explain the hypercholesterolaemia of casein-fed rabbits. J Nutr 113(3):618–629
24. Hu FB, Stampfer MJ, Manson JE et al (1999) Dietary protein and risk of ischemic heart disease in women. Am J Clin Nutr 70:221–227
25. Huff MW, Hamilton RMG, Carroll KK (1977) Plasma cholesterol levels in rabbits fed low fat, cholesterol-free, semipurified diets: effects of dietary proteins, protein hydrolysates and amino acid mixtures. Atherosclerosis 28(2):187–195
26. Huff MW, Roberts DCK, Carroll KK (1982) Long-term effects of semipurified diets containing casein or soy protein isolate on atherosclerosis and plasma lipoproteins in rabbits. Atherosclerosis 41:327–336
27. Jones RJ, Huffman S (1956) Chronic effect of dietary protein on hypercholesterolaemia in the rat. Proc Soc Exp Biol Med 93:519–522
28. Katan MJ, Louis HM, Vroomen LH, Hermus RJJ (1982) Reduction of casein-induced hypercholesterolaemia and atherosclerosis in rabbits and rats by dietary glycine, arginine and alanine. Atherosclerosis 43:381–391
29. Key TJ, Fraser GE, Thorogood M et al (1999) Mortality in vegetarians and nonvegetarians: detailed findings from a collaborative analysis of 5 prospective studies. Am J Clin Nutr 70:516S–524S
30. Kurowska EM, Carroll KK (1992) Effect of high levels of selected dietary essential amino acids on hypercholesterolaemia and down-regulation of hepatic LDL receptors in rabbits. Biochim Biophys Acta 1126(2):185–191
31. Kurowska EM, Carroll KK (1994) Hypercholesterolemic responses in rabbits to selected groups of dietary essential amino acids. J Nutr 124:364–370
32. Libby P (2002) Inflammation in atherosclerosis. Nature 420: 868–874
33. Libby P, Ridker PM, Maseri A (2002) Inflammation and atherosclerosis. Circulation 105:1135–1143
34. Little JM, Angell EA (1977) Dietary protein level and experimental aortic atherosclerosis. Atherosclerosis 26(2):173–179
35. Lofland HB, Clarkson TB, Goodman HO (1961) Interactions among dietary fat, protein, and cholesterol in atherosclerosis-susceptible pigeons: effect on serum cholesterol and aortic atherosclerosis. Circ Res 9:919–924
36. Lorient D, Linden G (1976) Dephosphorylation of bovine casein by milk alkaline phosphatase. J Dairy Res 43:19–26
37. Mahley RW, Holcombe KS (1977) Alterations of the plasma lipoproteins and apoproteins following cholesterol feeding in the rat. J Lipid Res 18:314–324
38. Meinhertz H, Nilausen K, Faergemen O (1990) Effects of dietary proteins on plasma lipoprotein levels in normal subjects: interaction with dietary cholesterol. J Nutr Sci Vitaminol 36(2):S157–S164
39. Moreno JJ, Mitjavila MT (2003) The degree of unsaturation of dietary fatty acids and the development of atherosclerosis. J Nutr Biochem 14(4):182–195
40. Nath N, Harper AE, Elvehjem CA (1959) Diet and cholesteremia: part 3 effect of dietary proteins with particular reference to the lipids in wheat gluten. Can J Biochem Physiol 37:1375–1384
41. Oakenfull DG, Fenwick DE, Hood RL (1979) Effects of saponins on bile acids and plasma lipids in the rat. Br J Nutr 42:209–216
42. Oakenfull DG, Sidhu GS (1990) Could saponins be a useful treatment for hypercholesterolemia? Eur J Clin Nutr 44:79–88
43. Sacks FM, Breslow JL, Wood PG, Kass EH (1983) Lack of an effect of dairy protein (casein) and soy protein on plasma cholesterol of strict vegetarians. An experiment and a critical review. J Lipid Res 24:1012–1020
44. Samman S, Khosla KK, Carroll KK (1990) Intermediate density lipoprotein-apolipoprotein B turnover in rabbits fed semipurified diets containing casein or soy protein. Ann Nutr Metab 34(2):98–103
45. Sindayikengera S, Xia W (2006) Nutritional evaluation of caseins and whey proteins and their hydrolysates from Protamex. J Zhejiang Univ (Sci) 7(2):90–98
46. Terpstra AHM, Harkes L, Van der Veen FH (1981) The effect of different proportions of casein in semipurified diets on the concentration of serum cholesterol and the lipoprotein composition in rabbits. Lipids 16:114–119
47. Terpstra AHM, Van Tintelin G, West CE (1981) The effect of semipurified diets containing different proportions of either casein or soybean protein on the concentrations of cholesterol in whole serum, serum lipoproteins and liver in male and female rats. Atherosclerosis 42:85–95
48. Van Der Meer R, De Vries H, Van Tintelen G (1988) The phosphorylation state of casein and the species-dependency of its hypercholesterolaemic effect. Br J Nutr 59:467–473
49. Van Raaij JMA, Katan MB, Hautvast JGAJ, Hermus RJJ (1981) Effects of casein versus soy protein diets on serum cholesterol and lipoproteins in young healthy volunteers. Am J Clin Nutr 34:1261–1271
50. Vega-Lopez S, Lichtenstein AH (2005) Dietary protein type and cardiovascular disease risk factors. Prev Cardiol 8(1):31–40
51. Xian CJ, Shoubridge CA, Read LC (1995) Degradation of IGF-1 in the adult rat gastrointestinal tract is limited by a specific antiserum or the dietary protein casein. J Endocrinol 146:215–225
52. Zhang X, Shu XO, Fao Y-T et al (2003) Soy food consumption is associated with lower risk of coronary heart disease in Chinese women. J Nutr 133:2874–2878

III

A low-carbohydrate high-protein diet induces vascular smooth muscle cell dedifferentiation in an ApoE-/- murine model

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Contribution of Authors

Olivia Hanna Koury: animal handling, surgeries, immunoblotting, measurements of contractility, analysis of data, preparation of manuscript.

Andreas Berdgahl: animal handling, concept development, student supervision, statistics, analysis of data, preparation of manuscript.

A low-carbohydrate high-protein diet induces vascular smooth muscle cell dedifferentiation in an ApoE-/- murine model

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Summary: Low-carbohydrate high-protein (LCHP) diets remain the leading weight loss regimen proving to be quite rapid and highly effective. These diets have been praised for the health benefits associated with insulin resistance, hypertension, weight loss, and lipid control. However, a link between these diets and altered vascular smooth muscle cell (VSMC) phenotype has yet to be addressed. With the rise in obesity, and frequent use of LCHP diets, this project aimed to investigate LCHP induced effects on the VSMCs. We hypothesized that the LCHP diets would experience a more significant shift to synthetic phenotype than a control (CON) or western diet (WD). Male Apolipoprotein E-deficient mice were randomly assigned to one of three diets: CON, WD, or LCHP diet. Following 6 weeks on the diet, animals were euthanized and their thoracic aortas were removed. Oil Red O staining, immunoblotting, and wire myography were performed on these aorta to assess the state of dedifferentiation of the VSMCs. Immunoblotting revealed a significant decrease of contractile proteins α-actin and calponin, and a significant increase of TRPC-1 indicating a shift from contractile to synthetic phenotype. This property did not translate to functional measures, as the wire myograph did not reveal a loss in force-generating capacity of the cell. Oil Red O staining did not show a major difference in lipid accumulation in the 3 diets. Data from this study revealed an evident dedifferentiation occurring in the VSMCs however this change did not manifest a change in the contractile ability of the cell.

Keywords: atherosclerosis • dedifferentiation• vascular smooth muscle cell •phenotype• low-carbohydrate high-protein

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Obesity has been associated with a host of adverse health conditions, such as insulin resistance, systemic inflammation and cardiovascular disease risk factors [1, 2]. Due to the deleterious effects of obesity on both society and the individual, the search for an efficient and safe means of weight loss remains a priority. Diets that are rich in protein and/or fat, with limited carbohydrates (i.e.: the Zone, Dr. Atkins) have earned attention due to their obvious potential for weight reduction. Despite their apparent "success", it remains to elucidate all potential side effects associated with these methods of weight loss. Recent data obtained in a murine model, highlights the possibility that not all diets are created "equal" and that the traditional serum risk markers used to assess weight loss programs may not capture the full picture. In the study by Foo *et al.*, (2009), Apolipoprotein E-knockout (ApoE-/-) mice given a low-carbohydrate high-protein (LCHP) diet (12% carbohydrate, 43% fat, 45% protein and 0.15% cholesterol) developed more aortic atherosclerotic lesions and impaired ability to generate new vessels in response to tissue ischemia when compared to both a

control group (regular chow diet of 65% carbohydrate, 15% fat and 20% protein) and, more interestingly, mice put on a representation of a western-type diet (43% carbohydrate, 42% fat, 15% protein) [3]. Although the data presented in the Foo *et al*. study is interesting in terms of the presence of atherosclerosis in the aorta, the link that they present between the low carbohydrate/high protein diet and the formation of lesions primarily rests on the evaluation of endothelial progenitor cells. These observations, however, do not take into consideration the stages in the development of atherosclerosis itself. Early atherosclerotic progression consists primarily of the infiltration of fat into the vascular wall and formation of fatty streaks and is reversible through dietary modifications, pharmacological interventions and exercise [4].

The following stages of atherosclerosis involve the development of the lipid core and stabilization of a fibrous cap, involving largely vascular smooth muscle cells (VSMCs) [5]. Differentiated VSMCs are highly specialized units, located in the media layer of arteries. Their primary function is to contract and relax, consequently controlling blood pressure and blood flow distribution [6-10]. In a healthy adult vessel, the contractile phenotype is characterized by markers such as smooth muscle (SM) α-actin, calponin, SM22-α, SM-myosin heavy chain, h-caldesmon [9]. Unique to VSMCs is their remarkable plasticity as they, unlike cardiomyocytes or skeletal muscle cells, are not terminally differentiated [9,11] and can thus modify their phenotype according to physiological provocations [11]. This shift from contractile to synthetic state serves great importance during vascular development and pathology as it allows for malleability and thus reversible adaptations [6, 10-12]. Following vascular injury, contractile VSMCs go through a transient modification of phenotype, which involves a reduced expression of contractile genes [10]. This plasticity seems to be necessary and may confer a survival advantage since it provides means for the smooth muscle to respond to altered conditions in its surroundings. The dedifferentiation of the VSMCs leads to migration and proliferation to the intimal layer, followed by the formation of connective tissue matrix that accumulates lipids and both free and esterified cholesterol [13]. At the point where vascular smooth muscle cells actually dedifferentiate and migrate into the vessel wall, the atherosclerotic lesions are considered no longer reversible and may present a serious concern in terms of cardiovascular risk [14]. However, the solid formation of extracellular matrix by VSMCs is also thought to serve as a protective component in plaque stabilization [13, 15] subsequently preventing rupture [16]. The situation is exacerbated by the continuous accumulation of macrophages that ingest lipids and become foam cells [17, 18]. At this stage, the response is thought to be chronic and the lesion progresses to a more advanced stage.

Foo et al as well as many other studies have looked at the effects of a low-carbohydrate diet in terms of insulin resistance, hypertension, ketosis, however, to date nothing has been produced about the VSMC phenotype switching [19-24]. The goal of this study was to determine whether the altered physiological environments induced by a low-carbohydrate diet would favor a VSMC phenotype shift.

Methods

Animals

Apolipoprotein E knockout (ApoE-/-) mice were obtained from Jackson Laboratories (Bar Harbor, Maine, USA) and used for breeding. The resulting litters were weaned and separated based on sex at 21-28 days. The males were housed individually and randomly assigned to one of three diets for 6 weeks: control (CON), western (WD), and low-carbohydrate high-protein (LCHP) diet. All procedures were approved by the Animal Ethics Committee of Concordia University (protocol ID: #30000259) and were conducted in accordance with guidelines of the Canadian Council on Animal Care.

Diet Specifications

All 3 animal diets were iso-caloric. Table 1 displays the diet specifications in terms of percentage of caloric intake from fat, carbohydrates, and protein. The CON diet, 5075 Charles River Autoclavable Rodent Diet, reflects a healthy, standard macronutrient distribution. The WD, obtained from Harlan Laboratories (TD.110229) replicates a high-fat high-

carbohydrate 'American' diet, consisting of 42% from fat, 42% from CHO, and 16% from protein. The LCHP diet, obtained from Harlan Laboratories (TD.04524) simulates an Atkin's diet used for weight loss and contains 43% from fat, 11% CHO, and 46% protein. Both WD and LCHP diets are modifications of TD.88137 (Harlan Laboratories), used for studies of atherosclerosis, and contain comparable amounts of cholesterol (1.5 g/kg).

Table 1. *Rodent diet specifications.*

	Control	Western	LCHP
Carbohydrates	63%	42.2%	11%
Fats	14%	42.1%	43.2%
Proteins	24%	15.7%	45.8%

Experimental Techniques

Oil Red O Staining

Oil Red O (ORO) staining, quantifying the lipid deposits within the vessel, was performed according to Nunnari *et al.* [25] on fresh tissue from the thoracic aorta. A 3% stock solution was prepared (10 ml isopropanol, 0.3g Oil Red O Powder from Sigma-Aldrich), as well as a working stain consisting of stock solution and water (3:2 parts respectively) filtered through a Whatman No. 1 Filter. The aortas were cleaned of adventitial fat and then soaked in the working stain for 30min, rinsed in distilled water, cleaned again of excess adventitial fat, and split longitudinally to measure for area. Samples were then individually placed in chloroform: methanol (1:1) solution for 2 min to extract the stain before the absorbance was measured at 520 nm using a spectrophotometer.

Immunoblotting

The thoracic aorta was removed from the sacrificed animal cleaned of fat and connective tissue, and stored at -80°C. The tissue was then pulverized/homogenized using liquid nitrogen with ~70µl lysis buffer containing (in mM) 250 NaCl, 50 HEPES (pH 7.5), 10% glycerol, 1% triton X-100, 1.5 $MgCl_2$ 1 EGTA, 10 $Na_4P_2O_7$ NaF, 800 µM Na_3VO_4. After 1 hour on ice, the cell slurry was centrifuged at 13,000 rpm for 10 min, and the supernatant was collected. 10µl of lysate was mixed with 2µl DTT and 2µl sample buffer and loaded on a 10% acrylamide-SDS gel followed by a transfer onto a 0.45µm nitrocellulose membrane (162-0115 Bio-Rad) in 10mM sodium tetraborate buffer. Ponceau staining was done as loading control before the membranes were blocked in 3% bovine serum albumin (BSA) in 0.1% Tween 20 in Tris-buffered saline (TBS) (10mM Tris-HCl, pH 7.5, 150 mm NaCl) for 1 hour at room temperature. This was followed by overnight incubation 4°C with Abcam primary antibodies: α-actin ab5694 (1:2000), calponin ab46794 (1:2000), TRPC1 ab88182 (1:2000). The membranes were then washed, and incubated with secondary antibodies: TRPC1 and calponin ab6721 (1:15000), α-actin ab6728 (1:15000). Membranes were exposed with ECL chemiluminescence and developed bands were analyzed with Image J Software.

Isometric Force

Vessels were dissected free of surrounding tissue and sliced into sections ~3mm in length. Sectioned vessels were mounted onto the Radnoti wire myograph working chamber with a 25µm Tungsten wire. Once securely mounted, the vessels were stretched to identical basal tonus in a Ca^{2+}-free Kreb's solution containing (in mM) 117.9 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 25 $NaHCO_3$, 1.2 NaH_2PO_4, 0.0027 EDTA, 0.1 ascorbic acid, 11 glucose. Once stable, 6 ml of HEPES buffered Kreb's solution containing (in mM) 135.5 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 11.6 glucose, 11.6 HEPES, pH 7.4 (NaOH) was added. When a stable baseline was attained the solution was changed to High K+ (HK) for approx. 6 min to induce maximal membrane depolarization and thus highest contraction. This process was repeated for a second High K+ contraction after sufficient relaxation. Following the second HK contraction and relaxation, endothelin-1 (ET-1) (Sigma Aldrich E7764) was added to the chamber giving final concentration of 10nM for approx. 5 min to generate a contraction. Data was analyzed using LabChart Reader version 8.0.

Statistical Analysis

Quantitative data are mean ± SEM. Statistical analysis was determined with 2-tailed Student's *t*-test or single-factor ANOVA. $p < 0.05$ was considered significant. n represents the sample number. In the figures presented, *p< 0.05, ** p< 0.01.

Results

Weekly Weight Gain Shows Peaks only in First week

Weekly weight was recorded over the 6 weeks of the study as shown in Figure 1. The first week on the respective diets translated to a significant difference in weight gain, with the WD showing a 58% increase, while the LCHP showed a 37% increase, and the CON a 39% increase (p< 0.01, n=11) from their baseline weight. However in the following weeks, there was no notable difference in weight gain amongst the three groups. The largest peak and difference in weight was therefore only seen in the beginning.

Lipid Accumulation is Quantified

To evaluate whether there was a meaningful accumulation of lipid in the thoracic aorta that would lead to an atherosclerotic lesion, an Oil Red O staining protocol was conducted. Recordings in Figure 2 show that despite trends, there was no significant difference of lipid deposition within the tissue in the WD and LCHP as compared to the CON (73.5%± 12.5 and 87.2%±15.5 respectively, p> 0.05, n= 6).

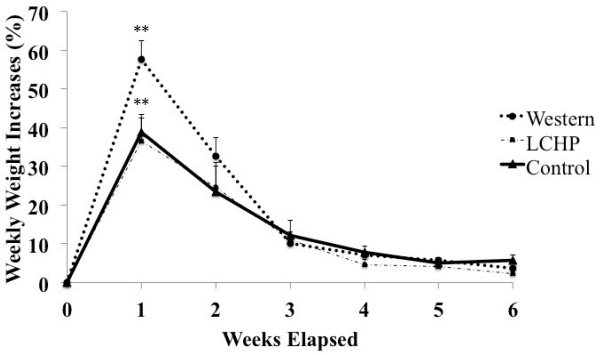

Figure 1. *Weekly weight increases show peaks in weight gain in first two weeks upon weaning, as well as a trend of lowest weight gain in the LCHP group, an expected outcome of this diet.*

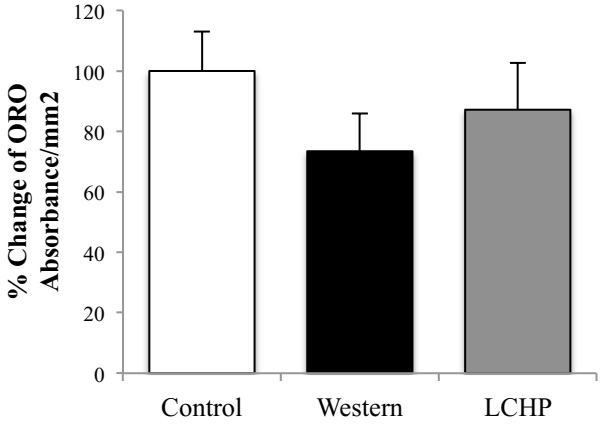

Figure 2: *ORO staining protocol revealed a non-significant trend in lipid deposition, relative to the control (73.5% ±12.5 and 87.2%± 15.5, p=0.39, n=6)*

Contractile Markers Show a Down-Regulation Paralleling Phenotype shift in VSMCs

To investigate how the various diets affected the expression of contractile markers, thoracic aortas were homogenized and western blot analysis revealed altered protein expression. As seen in Figure 3, the α-actin expression was significantly down regulated in the WD and LCHP as compared to the CON (89.6%± 4.7 and 79.5%± 7.6 respectively, p<0.05, n=9). Similarly, there was a significant down-regulation of calponin in the WD and LCHP as compared to the CON (87.7%± 6.1 and 71.4%± 6.1 respectively, p< 0.01, n=8). Synthetic phenotype would be marked by the significant increased expression of TRPC-1 channels in the WD and LCHP, also seen in Figure 3 (141.1%± 23.9 and 154.3%± 13.7 respectively, p< 0.05, n=8). There was no significant difference in any of the 3 markers between WD and LCHP diets, despite consistent trends showing a greater reduction in LCHP.

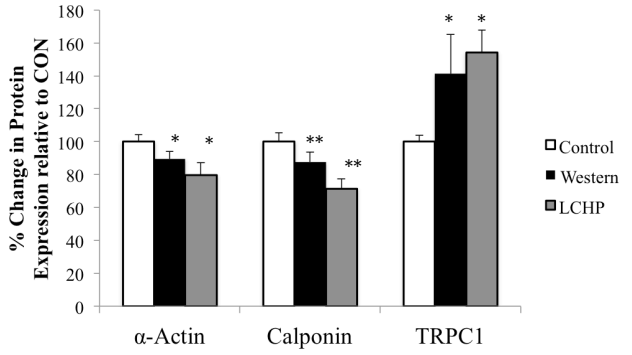

Figure 3. *Immunoblotting shows a significant trend of dedifferentiation occurring by a decreased expression of α-actin compared to CON (89.6%± 4.7 and 79.5%± 7.6,p= 0.047, n= 9), decreased expression of calponin compared to CON (87.7%± 6.1 and 71.4%± 6.1, p= 0.0088, n= 8), and increased expression of TRPC1 compared to CON (141.1%± 23.9 and 154.3%± 13.7, p= 0.033, n=8).*
Error bars represent SEM.

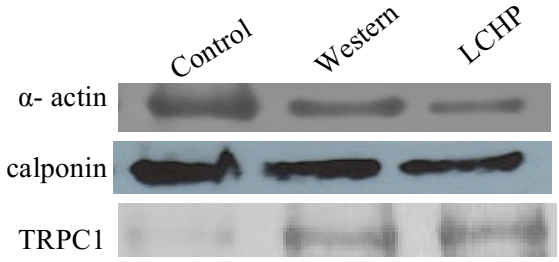

Figure 4: *Representative protein gels of immunoblotting*

VSMC Dedifferentiation did not Translate to a Deficit in Functional Capacity

To further examine the loss of contractile phenotype in the cell, the contractile ability was investigated. For this, 2-3mm aortic segments were suspended in a Radnoti wire myograph working chamber. There was a negligible difference between the maximal force generated by the 3 groups (CON, WD, and LCHP) following depolarization of the cell membrane by High-K saline solution (100%± 7.2, 106.6%± 15.8, 106.3%± 9.0, respectively, p>0.05, n=12). Furthermore, we investigated the reactivity of the VSMCs to ET-1, and the induced contraction relative to the HK tension generated was once again comparable between the 3 groups CON, WD, and LCHP (42.2%±7.0, 42.7% ± 3.4, 42.7% ± 3.2 respectively, p>0.05, n=12).

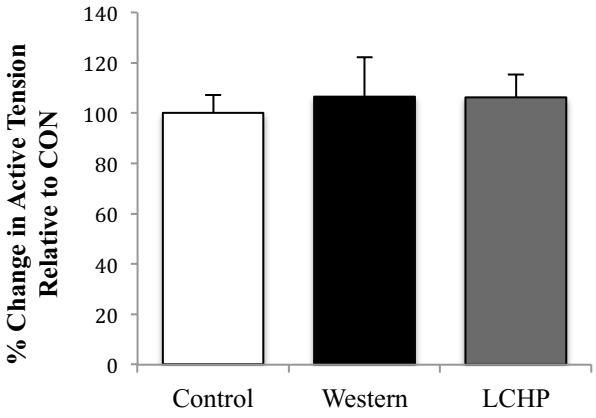

Figure 5. *There is no difference in force generated following HK membrane depolarization in the WD and LCHP diet, compared to CON (106.6% ± 15.8, 106.3%± 9, ± 0.018 respectively, p= 0.92, n=12).*

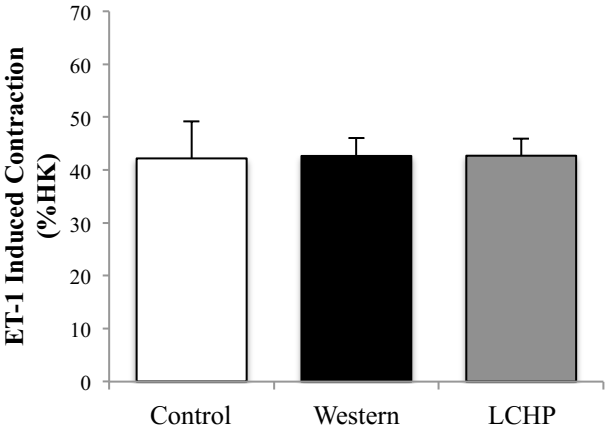

Figure 6. *There is negligible difference in the vasoreactivity to ET-1 compared to the HK, between the 3 groups (42.2%±7.0, 42.7% ± 3.4, 42.7% ± 3.2 respectively, p=0.99, n=12)*

Discussion

A pilot study was performed by Foo and colleagues using the same 3 diets as in this study, showing an increased percentage of atheroma in the aorta of animals on the LCHP diet compared to the WD and CON [3]. This finding raised the following question: if significantly higher amounts of atherosclerotic plaque develop in LCHP animals compared to the CON and WD, the activity of the VSMCs must be altered in a manner promoting cell dedifferentiation.

The main finding from this study was the evident shift of phenotype from contractile to synthetic within the VSMCs. Evaluating the state of the mature vessel depends on the relative presence of a variety of contractile apparatus proteins such as SM α-actin [26-28] and calponin [29-31], amongst others. It is evident that α-actin plays a vital role in cell contraction and is required for force-generating capacity of the VSMC [9, 32, 33] In murine aortic SM, 94% of the SM-actin is found in the α-type [34]. Similarly α-actin comprises >25% of the *total* protein in differentiated VSMCs proving to be the single most abundant protein in these matured cells [34-36]. It is well accepted that this predominant form SM α-actin, is greatly reduced following endothelial injury, and could represent as low as only 10% of the total SM-actin protein, formerly 94% [26, 37]. Our data demonstrated an approximate 20% reduction of α-actin in the LCHP from the control group, and 10% reduction in the WD. In cell differentiation, SM α-actin gene is activated early in embryonic development, and expressed early in the SM lineage [36, 38-42].

Another traditional contractile marker is calponin, first discovered in 1986 by Takahashi and colleagues [43]. Calponin, a calcium regulatory protein, is involved in regulation of SM contraction [10]. Calponin interacts with actin and tropomyosin in a Ca^{2+}-independent manner, and calmodulin in a Ca^{2+}-dependent manner [43, 44]. The amounts of calponin were decreased by approximately 30% in the LCHP and around 12% in the WD relative to the control group, conceptualizing the idea of loss of contractile property. Calponin is known to be expressed as an intermediate marker in the differentiation and dedifferentiation of SMC [29].

In addition to contractile proteins to mark an altered phenotype, other changes such as membrane properties, receptor population, and Ca^{2+} control might ensue prior to phenotype modulation [45]. Ca^{2+} handling and manipulation can have a gross impact on resultant cellular contraction, wherein addition of extracellular Ca^{2+} channels can have a profound proliferative effect [46, 47]. T-type calcium channels, more specifically the TRPC-1 type [48, 49], have been linked to store-operated Ca^{2+} entry (SOCE), which has been found to be up-regulated in proliferating SMCs of the pulmonary artery [50]. Kumar and colleagues established that calcium channel TRPC-1 is commonly increased in blood vessels as a compensatory protective response following vascular injury [51]. This fact parallels the shift to synthetic previously described, since there was a 54% increase of TRPC1 in the LCHP and 41% increase in the WD compared to the CON group.

Since we observed a modulation of contractile proteins in the VSMCs, we questioned whether this alteration was provoked by an increase in lipid or some other endogenous factor. Although atherosclerosis is now understood to be more of an inflammatory disease, rather than a lipid-burdened disease [52], we nevertheless wanted to assess whether an excess of lipid may have played a role. An ORO staining protocol was used to quantify the concentration of lipid residing in the thoracic aorta. Interestingly enough, there was less lipid content in the WD and LCHP compared to the CON, however these trends were not significant.

Following evidence of a phenotype shift, the functional capacity of the cell was examined by means of wire myography. Whereby the pressure myography is favorable for small vessels with significant vasoreactivity, the wire myograph is a sensitive method preferred for larger vessels and records the tension produced under isometric conditions [53]. The aorta was sectioned and tested using High-K^+ saline and endothelin-1 (ET-1) solution, predicted to demonstrate a decrease in tension or force produced. ET-1 is a potent vasoconstrictor and pro-inflammatory peptide [54]. Contrary to our expected outcome, the data revealed no difference in either case of induced contraction from the High-K solution or vasoconstrictor ET-1. The explanation for this observation may be two-fold. From one perspective, the 6-week length of study may have been premature to witness an impairment of vascular contractility. Contractile proteins α-actin and calponin are early and intermediate markers of dedifferentiation respectively, and only provide grounds to confirm events occurring at a certain point in time: early to intermediate dedifferentiation [29, 38]. Perhaps, functional capacity is only affected during late dedifferentiation. Lesions containing foam cells and VSMCs are typically seen at

8-10 weeks old in an ApoE -/- mouse given normal chow [55, 56] however, we noted a change in VSMC after 6 weeks of diet (approximately 9 weeks old). Fibrous plaques tend to appear at 15-20 weeks of age [55, 56]. It may have been an option to extend the 6 weeks, and see if the increased time translated to a subsequent loss in contractile response. On the other hand, it could be speculated that the thoracic aorta may never exhibit impressive contraction, as it is not physiologically responsible for maintenance of vascular tone and high vasoreactivity. Nonetheless, this is not a sound argument since there are studies that investigate vascular contractility in the aorta [36, 57]. Complete loss in contractile response following depolarization with KCl is possible, and has been shown using control and α-actin null mice [36], displaying a fundamental dysfunction in the contractile system with alteration in SM α-actin genes.

Data from this study show that a particular diet low in carbohydrates and high in protein, offers an imbalanced environment that favors a synthetic phenotype in VSMCs. Macronutrient composition is altered in a LCHP diet, consequently affecting the fuel sources and cellular metabolism. Although the same energy sources are utilized, the majority of the energy is now derived from fatty acids and ketones, hence an 'adipocentric' energy source as opposed to a 'glucocentric' source [58]. LCHP diets are frequently used for rapid and efficient weight loss, and this is conceptualized by the fact that LCHP animals did not gain as much weight as the WD (37% and 58%, respectively) in the beginning weeks, as seen in Figure 1. In a LCHP diet, approximately 70% of caloric requirements come from fatty acids via dietary fat or lipolysis, 20% come from ketone bodies acetoacetate and β-hydroxybutyrate, and 10% from glucose via gluconeogenesis or glycogenolysis [59]. This adaptation is of no surprise; it is thought that a substrate or by-product of using such energy sources plays an indirect role in mediating phenotype shift in VSMCs.

Platelet-derived growth factor-BB (PDGF-BB) is well documented as being responsible for some phenotype regulation, since it is a chemotactic agent meaning, a molecule that favors migration and proliferation [60-63]. Although there is a link with PDGF and suppressed marker genes, another very influential factor is the gene known for maintenance of pluripotent embryonic stem cells: Kruppel-like Factor 4 (KLF4) [64]. Not typically expressed in adult mesenchymal cells [65], KLF4 becomes re-expressed and plays a role in mediating SMC phenotype switching *in vivo* [60, 66], since it strongly represses expression of SMC genes [60]. Following vascular injury, ApoE -/- mice on a WD showed increased expression of KLF4 [67, 68] and this abundance of KLF4 is accompanied by an inhibition of expression of known SMC marker genes [69]. KLF4 also has the ability to inhibit the increase of SMC differentiation mediated by TGF-β [14]. Taken together, this provides reason to speculate whether KLF4 can serve as a plausible endogenous factor indirectly involved in the VSMC activity through growth factors however, this theory lies beyond the scope of this study.

Conclusion

This study aimed to assess the effects of a low-carbohydrate high-protein diet on the role of vascular smooth muscle cells in atherosclerosis. Prior to our study, a possible link between VSMC phenotype and LCHP diets had never been made. This study emphasized how such a topic does indeed merit further enquiry, as there is a clear dedifferentiation occurring in VSMCs of ApoE -/- mice on a LCHP diet, potentially providing intriguing or relevant data for clinicians and patients both interested in this type of diet.

References

1. 1. Grundy SM (2004) Atherosclerosis imaging and the future of lipid management. *Circulation.* Vol 110(23): 3509-3511.

2. Dandona P, Aljada A, Chaudhuri A, et al. (2005) Metabolic syndrome: a comprehensive perspective based on interactions between obesity, diabetes, and inflammation. *Circulation.* Vol 111(11) 1448-1454.

3. Foo SY, Heller ER, Wykrzykowska J, Sullivan CJ, Manning-Tobin JJ, Moore KJ, […] Rosenzweig A (2009) Vascular effects of a low-carbohydrate high-protein diet. *Procl Nat Acad Sci.* Vol 106(36)15418-15423.

4. Scott JJ (2002) The pathogenesis of atherosclerosis and new opportunities for treatment and prevention. *J of Neural Transmission-Supplement.* 63:1-17.

5. Ross R and Glomset JA (1976) The pathogenesis of atherosclerosis. *N Engl J Med* 295: 369 –377/420 – 425.

6. Rzucidlo EM, Martin KA, Powell RJ (2007) Regulation of vascular smooth muscle cell differentiation. *J of Vasc Surg.* 45: 25A-32A.

7. Albinsson S, Hellstrand P (2007) Integration of signal pathways for stretch-dependent growth and differentiation in vascular smooth muscle. *Am J Physiol Cell Physiol.* 293: C772-782.

8. Hayashi K et al. (2001) Phenotypic modulation of vascular smooth muscle cells induced by unsaturated lysophosphatidic acids. *Circ Res.* 89: 251-258.

9. Owens GK (1995) Regulation of Differentiation of Vascular Smooth Muscle Cells. *Physiol. Rev.* 75(3) 487-517.

10. Owens GK, Kumar MS, Wamhoff BR (2004) Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev.* 84: 767-801.

11. Zheng J-P, Ju D, Shen J, Yang M, Li L (2010) Disruption of actin cytoskeleton mediates loss of tensile stress induced early phenotypic modulation of vascular smooth muscle cells in organ culture. *Exp and Molecul Pathol.* 88: 52-57.

12. Pipes GC et al. (2006) The myocardin family of transcriptional coactivators: versatile regulators of growth, migration, and myogenesis. *Genes Dev.* 20: 1545-1556.

13. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s (1993) *N Engl J Med* 362: 801–809

14. Ross R (1999) Atherosclerosis- an inflammatory disease. *N Engl J Med.* 340: 115-126.

15. Ross R. The pathogenesis of atherosclerosis—an update (1986) *N Engl J Med* 314: 488 –500.

16. Galis ZS, Sukhova GK, Lark MW, Libby P (1994) Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions in human atherosclerotic plaque. *J Clin Invest.* 94: 2493-2503.

17. Hansson GK, Jonasson L, Seifert PS, et al. (1989) Immune-mechanisms in atherosclerosis. *Arteriosclerosis.* Vol 9(5): 567-578.

18. Roselaar SE, Kakkanathu PX, Daugherty A (1996) Lymphocyte populations in atherosclerotic lesions of ApoE -/- and LDL receptor -/- mice- decreasing density with disease progression. *Arterioscler Thromb Vasc Biol.* Vol 16(8) 1013-1018.

19. Boden G, Sargrad K, Homko C, Mozzoli M, Stein TP (2005) Effect of a low-carbohydrate diet on appetite, blood glucose levels, and insulin resistance in obese patients with type-2 diabetes. *Annals of Internal Medicine.* 142(6): 403-411.

20. Cornier, MA., Donahoo, WT., Pereira, R., Gurevich, I., Westergren, R., Enerback, S., …Draznin, B. (2005). Insulin sensitivity determines the effectiveness of dietary macronutrient composition on weight loss in obese women. *Obes Res.* 13: 703-709.

21. Samaha FF, Iqbal N, Seshadri P, et al. (2003) A low-carbohydrate as compared with a low-fat diet in severe obesity. *NEJM.* Vol 348(20) 2074-2081.

22. Linn, T., Santosa, B., Gronemeyer, D., Aygen, S., Scholz, N., Busch, M. & Bretzel, RG. (2000). Effects of long term dietary protein intake on glucose metabolism in humans. *Diabetalogia.* 43: 1257-1265.

23. Foster, GD., Wyatt, HR., Hill, JO., McGuckin, BG., Brill, C., Mohammed, BS., … Klein, S. (2003). A randomized trial of a low-carbohydrate diet for obesity. *N Engl J Med.* 348: 2082-2090.

24. Bilsborough, SA. & Crowe, TC. (2003). Low-carbohydrate diets: what are the potential short- and long-term health implications? *Asia Pacific J Clin Nutr.* 12(4): 396-404.

25. Nunnari JJ, Zand T, Joris I, Majno G (1989) Quantitation of Oil Red O Staining of the aorta in hypercholesterolemic rats. *Exp and Molec Pathol..* 51: 1-8.

26. Gabbiani G. Kocher O, Bloom WS (1984) Actin expression in smooth muscle cells or rat aortic intimal thickening, human atheromatous plaque, and cultured rat aortic media. *J Clin Invest.* 73: 148-152.

27. Hungerford JE, Owens GK, Argraves WS, and Little CD (1996) Development of the aortic vessel wall as defined by vascular smooth muscle and extracellular matrix markers. *Dev Biol* 178: 375–392.

28. Mack CP and Owens GK (1999) Regulation of SM alpha-actin expression in vivo is dependent upon CArG elements within the 5' and first intron promoter regions. *Circ Res* 84: 852–861.

29. Duband JL, Gimona M, Scatena M, Sartore S, and Small

JV (1993) Calponin and SM 22 as differentiation markers of smooth muscle: spatiotemporal distribution during avian embryonic development. *Differentiation* 55: 1–11.

30. Miano JM, Cserjesi P, Ligon K, Perisamy M, and Olson EN (1994) Smooth muscle myosin heavy chain marks exclusively the smooth muscle lineage during mouse embryogenesis. *Circ Res* 75: 803– 812.

31. Strasser P, Gimona M, Moessler H, Herzog M, and Small JV (1993) Mammalian calponin: identification and expression of genetic variants. *FEBS Lett* 330: 13–18.

32. Kim HR, Gallant C, Leavis PC, Gunst SJ, Morgan KG (2008) Cytoskeletal remodeling in differential vascular smooth muscle is actin isoform dependent and stimulus dependent. *Am J Physiol Cell Physiol.* 295:C768-C778.

33. Owens GK, Thompson MM (1986) Developmental changes in isoactin expression in rat aortic smooth muscle cells in vivo. Relationship between growth and cytodifferentiation. *J Biol Chem.* 261(28): 13373-13380.

34. Vandekerckhove J and Weber K (1981) Actin typing on total cellular extracts. *Eur J Biochem.* 113: 595-603.

35. Fatigati V and Murphy RA (1984) Actin and tropomyosin variants in smooth muscles: dependence on tissue type. *J Biol. Chem.* 259:14383-14388.

36. Schildmeyer LA, Braun R, Taffet G, Debiasi M, Burns AE, Bradley A, Schwartz RJ (2000) Impaired vascular contractility and blood pressure homeostasis in the smooth muscle α-actin null mouse. *FASEB Journal.* 14: 2213-2220.

37. Kocher O, Skalli O, Bloom WS, Gabbiani G (1984) Cytoskeleton of rat aortic smooth muscle cells. Normal conditions and experimental intimal thickening. *Lab Invest.* 50(6): 645-652.

38. Owens GK, Loeb A, Gordon D, Thompson MM (1986) Expression of smooth muscle specific α-isoactin in cultured vascular smooth muscle cells: relationship between growth and cytodifferentiation. *J Cell Biol.* 102(2): 343-352.

39. Ruzicka DL & Schwartz RJ (1988) Sequential activation of α-actin genes during avian cardiogenesis: vascular smooth muscle α-actin gene transcripts mark the onset of cardiomyocyte differentiation. *J. Cell. Biol.* 107: 2575-2586.

40. McHugh KM, Crawford K, Lessard JL (1991) A comprehensive analysis of the developmental and tissue specific expression of the isoactin multigene family. *Dev. Biol.* 148: 442-458.

41. Sugi Y and Lough J (1992) Onset of expression and regional deposition of alpha-smooth and sarcometric actin during avian heart development. *Dev. Dynamics*. 193: 116-124.

42. Babai F, Musevi-Aghdam J, Schurch W, Royal A, Gabbiani G (1990) Coexpression of α-sarcometric actin, α-smooth muscle actin and desmin during myogenesis in rat and mouse embryos, in skeletal muscle. *Differentiation.* 44:132-142..

43. Takahashi K, Hiwada K, Kokubu T (1986) Isolation and characterization of a 34000Da calmodulin- and F-actin binding protein from chicken gizzard smooth muscle. *Biochem Biophys Res Commun.* 141:20-26.

44. Winder SJ & Walsh MP (1990) Smooth muscle calponin. Inhibition of actomyosin MgATPase and regulation by phosphorylation. *J Biolog Chem.* Vol 265(17): 10148-10155.

45. Bergdahl A, Gomez MF, Wihlborg A-K, Erlinge D, Eyjolfson A, Shang-Zhong X, Beech DJ, Dreja K, Hellstrand P (2005) Plasticity of TRPC expression in arterial smooth muscle: correlation with store-operated Ca^{2+} entry. *Am J Physiol Cell Physiol.* 288: C872-880.

46. Magnier-Gaubil C, Hebert JM, Quarck R, Papp B, Corvazier E, Wuytack F, Levy-Toledano S, Enouf J (1996) Smooth muscle cell cycle and proliferation. Relationship between calcium influx and sarco-endoplasmic reticulum Ca^{2+} ATPase regulation. *J Biol Chem.* 271:27788-27794.

47. Munaron L, Antoniotti S, Fiorio Pla A, Lovisolo D (2004) Blocking Ca^{2+} entry: a way to control cell proliferation. *Curr Med Chem.* 11: 1533- 1543.

48. Wes PD, Chevesich J, Jeromin A, Rosenberg C, Stetten G, Montell C (1995) TRPC1, a human homolog of Drosophila store-operated channel. *Proc Natl Acad Sci USA.* 92: 9652-9656.

49. Xu SZ, Beech DJ (2001) TRPC1 is a membrane-spanning subunit of store-operated Ca^{2+} channels in native vascular smooth muscle cells. *Circ Res.* 88:84-87.

50. Golovina VA, Platoshyn O, Bailey CI, Wang J, Limsuwan A, Sweeney M, Rubin LJ, Yuan JX (2001) Upregulated TRP and enhanced capacitative Ca^{2+} entry in human pulmonary artery myocytes during proliferation. *Am J Physiol Heart Circ Physiol.* 280: H746-H755.

51. Kumar B, Dreja K, Shah SS, Cheong A, Xu S-Z, Sukumar P […] Beech DJ (2006) Upregulated TRPC1 channel in vascular injury in vivo and its role in human

neointimal hyperplasia. *Circ Res.* 98: 557-563.

52. Libby P, Ridker PM, Maseri A (2002) Inflammation and atherosclerosis. *Circulation.* 105:1135-1143.

53. Lu X & Kassab GS (2007) Vasoreactivity of blood vessels using a novel isovolumic myograph. *An Biomed Eng.* 35: 356-366.

54. Bohm F & Pernow J (2007) The importance of endothelin-1 for vascular dysfunction in cardiovascular disease. *Cardiovascular Research*. 76: 8-18.

55. Plump AS, Smith JD, Hayek T, Aalto-Setala K, Walsh A, Verstuyft JG, Rubin EM, Breslow JL (1992) Severe hypercholesterolemia and atherosclerosis in apolipoprotein A-deficient mice created by homologous recombination in ES cells. *Cell.* 71(2) 343-353.

56. Nakashima Y, Plump AS, Raines EW, Breslow JL, Ross R (1994) ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. *Arterioscler Thromb.* 14(1) 133-140.

57. Moltzer E, Riet L, Swagemakers SMA, Heijningen PM, Vermeij M, van Vegnel R […] Essers J (2011) Impaired vascular contractility and aortic wall degeneration in Fibulin-4 deficient mice: effect of angiotensin II type 1 (AT_1) receptor blockade. *PLOS one.* Vol 6(8): 1-12.

58. Westman EC, Mavropoulos J, Yancy WS, Volek JS (2003) A review of low-carbohydrate ketogenic diets. *Current Atherosclerosis Reports.* 5:476-483.

59. Westman EC, Feinman RD, Mavropoulos JC, Vernon MC, Volek JS, Wortman JA, Yancy WS, Phinney SD (2007) Low-carbohydrate nutrition and metabolism. *Am J Clin Nutr.* 86:276-284.

60. Yan F-F, Liu Y-F, Liu Y, Zhao Z-X (2007) KLF4: a novel target for the treatment of atherosclerosis. *Medical Hypotheses.* 70: 845-847.

61. Berman, JW, Kazimi, M., Ma, H. (2002). Development of the Atherosclerotic Plaque. In D.L. Brown (Ed.), *Cardiovascular Plaque Rupture* (pp. 1-50). New York, NY: Marcel Dekker.

62. Holycross BJ, Blank RS, Thompson MM, Peach MJ, and Owens GK (1992) Platelet-derived growth factor-BB-induced suppression of smooth muscle cell differentiation. *Circ Res* 71: 1525–1532.

63. Wang Z, Wang DZ, Hockemeyer D, McAnallyJ, Norheim A, Olsen EN (2004) Myocardin and ternary complex factors compete for SRF to control smooth muscle gene expression. *Nature.* 428: 185-189.

64. Jiang J, Chan YS, Loh YH, Cai J, Tong GQ, Lim CA, et al. (2008) A core Klf circuitry regulates self-renewal of embryonic stem cells. *Nat Cell Biol.* 10:353-360.

65. Gimbrone MA, Topper JN, Nagel T, et al. (2000) Endothelial dysfunction, hemodynamic forces, and atherogenesis. *Ann NY Acad Sci.* 902:230-239.

66. Gomez D and Owens GK (2012) Smooth muscle cell phenotypic switching in atherosclerosis. *Cardiovascular Research.* 95:156-164.

67. Cherepanova OA, Pidkovka NA, Yoshida T, Gan Q, Adiguzel E, Bendeck MP, et al. (2009) Oxidized phospholipids induce type VIII collagen expression and vascular smooth muscle cell migration. *Circ Res.* 104: 609-618.

68. Yoshida T, Kaestner KH, Owens GK (2008) Conditional deletion of Kruppel-like factor 4 delays downregulation of smooth muscle cell differentiation markers but accelerates neointimal formation following vascular injury. *Circ Res.* 102: 1548-1557.

69. Liu Y, Sinha S, McDonald OG, Shang Y, Hoofnagle MH, Owens GK (2005) Kruppel-like factor 4 abrogates myocardin-induced activation of smooth muscle gene expression. *J Biol Chem.* 280:9719-9727.

IV

Altered mitochondrial functioning in apolipoprotein E-deficient mice induced by a low-carbohydrate high-protein diet

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Contribution of Authors

**Olivia Hanna Koury:* animal handling, surgeries, immunoblotting, analysis of data, preparation of manuscript.

**Cynthia Rocha:* high-resolution respirometry measurements, analysis of data, preparation of manuscript.

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Altered mitochondrial functioning in apolipoprotein E-deficient mice induced by a low-carbohydrate high-protein diet

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Summary: Low-carbohydrate high-protein (LCHP) diets have been growing in popularity in America for decades, proving to be one of the most popular and efficient methods of weight loss. Despite the superficial health benefits this type of diet may contribute in the case insulin resistance, hypertension, and lipid control for example, its implications on mitochondrial oxidative capacity and respiration have yet to be evaluated. The goal of this study was to evaluate the safety of LCHP diets by determining whether they foster an environment altering mitochondrial functioning. Male Apolipoprotein E-deficient mice were randomly assigned to either a control diet or low-carbohydrate high-protein diet for 6 weeks. Heart tissue was used for immunoblotting, and high-resolution respirometry (HRR) to quantify mitochondrial complexes and measure oxidative phosphorylation (OXPHOS) capacity. Our results indicate increased expression of all five mitochondrial complexes in the LCHP group compared to the control. Furthermore, HRR revealed increased efficiency of substrate utilization implying improved mitochondrial respiration. These findings correlate well with the understanding that cardiomyocytes prefer lipid as a fuel source, and the shift in metabolism caused by a LCHP diet provides such a favourable environment.

Keywords: Apolipoprotein E • cardiac mitochondria• oxidative capacity •bioenergetics• low-carbohydrate high-protein

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The recent rise in obesity has been associated with a number of adverse health effects such as heart disease, certain types of cancer, type-2 diabetes mellitus, and respiratory complications [1]. In efforts to reduce associated health risks, overweight and obese patients have been advised to maintain a healthy BMI. One of the most efficient means of weight loss is through dieting, more specifically a low-carbohydrate high-protein diet (LCHP). This diet has gained much popularity with the American public [2], and advocates claim it 'burns more fat' and leads to quicker weight loss without adverse long-term effects [3]. Macronutrient composition is altered in a LCHP diet, drastically affecting the cellular metabolism. Although the same energy sources are utilized, majority of the energy is now derived from fatty acids and ketones due to the extreme reduction of carbohydrate. This is shifts metabolism from a 'glucocentric' source to an 'adipocentric' energy source [4]. In LCHP metabolism, 70% of caloric requirements come from fatty acids via dietary fat or lipolysis, 20% come from ketone bodies acetoacetate and β-hydroxybutyrate, and 10% from

glucose via gluconeogenesis or glycogenolysis [5]. LCHP diets have been studied extensively in the past decades regarding weight loss, lipid markers, insulin sensitivity, hypertension, and endothelial dysfunction [6-11]. However, no study has addressed a possible link with LCHP diets and heart failure due to cardiac mitochondrial dysfunction.

Despite a general decrease in the number of deaths from cardiovascular disease (CVD) in the United States, congestive heart failure (CHF) remains as prevalent today as it was two decades ago [12]. Although very broadly defined, CHF can be best described as a multifactorial syndrome that renders the myocardium unable to pump blood efficiently to sustain demand [13]. Oxidative stress is now understood to be a key factor in heart failure, and a main reason for oxidative stress is the imbalance and negative impact of reactive oxygen species (ROS) produced by the mitochondria [14-18]. In recent years, studies have linked characteristics of heart failure to defective mitochondrial energetics and abnormal substrate metabolism [19- 21]. There is significant evidence showing mitochondrial dysfunction not only in cardiomyocytes, but also skeletal muscle of heart failure patients [22]. Aside from mitochondrial dysfunction, other problems that may contribute to the noticeable energetic defects are the number of mitochondria, substrate of choice, or oxidative capacity [23]. These facts imply that preserving cardiac mitochondrial functioning can become a future therapeutic aim for CHF [24-26].

Mitochondria are potentially significant sources of ROS especially when damaged (i.e. by long-term FA accumulation,) where they exhibit decreased oxidative based energy and thus less fuel for cells [27]. This is a central concern since the mitochondrion is the organelle in which energy (ATP) production and cellular respiration occur [28, 29]. Considering the maintenance of ATP drives all cellular processes, this process is imperative for a high-level workload organ such as the heart due to its high-energy demands [25, 30-33]. The capacity of the mitochondria relies heavily on substrate availability, notably lipids and carbohydrates. These substrates contribute to energy metabolism depending on their utilization through β-oxidation and the Kreb's cycle. β-oxidation produces acetyl-CoA from FAs that enters the Kreb's cycle, generating electrons that are successively transported to the electron transport system (ETS) by NADH and $FADH_2$ to amplify ATP production [34]. The Kreb's cycle is the focal common aerobic metabolic pathway for carbohydrates, lipids and proteins [35]. Acetyl-CoA oxidation by this cycle is responsible for two thirds of the sum of ATP production and oxygen consumption [36].

Despite glucose and FAs both being oxidized in the mitochondria to ATP production in the cardiomyocytes, the latter is the preferred substrate (generating 70% of total ATP) [37-39]. Therefore, the uptake of FA from the plasma is vital for cardiac viability [21, 37- 40]. Furthermore, lipids have also been deemed necessary for the maintenance and re-initiation involved with the pure beating of heart cells, further highlighting their significance [41]. The heart has limited substrate storage capacity, thus the uptake of nutrients needs to be finely balanced since the underlying pathways have to respond both appropriately and competently to the continuous flux in energy demand and substrate availability [25]. Under normal conditions the mitochondria maintain a fine equilibrium between glucose and FAs, however, during physiological stress, the mitochondria shifts towards an increased carbohydrate metabolism. This shift adds stress to the heart and shunts the lipids into non-oxidative pathways creating more ROS than energy [42]. The activity of respiratory complex I in the mitochondria is especially prone to the effects of oxidative damage [43]. The same detrimental phenomenon occurs when there is a reduced oxygen supply as a result of the occlusion of the coronary vessels, a major cause of cardiovascular distress [44].

The mitochondria are an important health research target considering their role in generating cellular energy, by means of oxidative phosphorylation, a process affected by uptake of cholesterol and lipids from the plasma. The subsequently altered role of mitochondria may potentially be implicated in the pathophysiology of heart failure [45] and overall cardiovascular health. The apolipoprotein E deficient (ApoE-/-) murine model is a well-established model as it allows for the investigation of atherosclerosis due to its susceptibility to develop lesions rapidly, and

similarly to humans [46-48]. ApoE is a 34 kDa glycoprotein made in the liver and brain, responsible for lipoprotein metabolism [48]. It helps to clear circulating cholesterol by mediating the binding of ApoE-containing lipoproteins and LDL receptors [49-52].

This study examined whether ApoE-/- mice exhibit altered cardiac mitochondrial oxidative capacity when exposed to a low-carbohydrate high-protein environment. We quantified cardiac mitochondrial consumption in a 6-week old ApoE-/- murine model in comparison to age-matched controls. The results determined if alterations in energy metabolism had occurred by examining the oxidative capacity of the mitochondrial respiratory complexes. We hypothesized that the mitochondria from the ApoE-/- cardiac tissue would experience increased respiratory function and disturbed energy metabolism as compared to controls.

Methods

Animal Care

Apolipoprotein E knockout (ApoE-/-) mice were obtained from Jackson Laboratories (Bar Harbor, Maine, USA) and used for breeding. The resulting litters were weaned and separated based on sex at 21-28 days. Only the tissue from male mice was used for the purpose of this study. The males were housed individually in a thermo-neutral environment (22ºC), on a 12:12 h photoperiod, and randomly assigned to either a control diet (CON) or a low-carbohydrate high-protein (LCHP) diet for 6 weeks. Both groups of mice had access to water and their respective diets ad libitum. All procedures were approved by the Animal Ethics Committee of Concordia University (protocol ID: #30000259) and were conducted in accordance with guidelines of the Canadian Council on Animal Care.

Diet Specifications

Both CON and LCHP animal diets were iso-caloric. Table 1 displays the diet specifications in terms of percentage of caloric intake from fat, carbohydrates (CHO) and protein. The CON diet, 5075 Charles River Autoclavable Rodent Diet, reflects a healthy, standard macronutrient distribution. Whereas, the LCHP diet, obtained from Harlan Laboratories (TD.04524) simulates an Atkin's diet used for weight loss and contains 43% from fat, 11% CHO, and 46% protein. The LCHP diet is a modification of TD.88137 (Harlan Laboratories), used for studies on atherosclerosis.

Experimental protocol

The beating heart was removed immediately after euthanasia with CO_2 according to the approved animal protocol and split into two different portions. One portion was snap frozen in liquid nitrogen, and stored at -80ºC for biochemical analysis; the other portion (the apex) was placed in an ice cold relaxing buffer (BIOPS) and used immediately to measure mitochondrial respiration. The BIOPS contains (in mM): CaK_2EGTA 2.77, K_2EGTA 7.23, Na_2ATP 5.77, $MgCl_2 \cdot 6H_2O$ 6.56, Taurine 20, Na_2Phosphocreatine 15, Imidazole 20 mM, Dithiothreitol 0.5, MES 50, pH 7.1

Preparation of permeabilized cardiac fibers

The apex was dissected for preparation of permeabilized myofibers. This was done by gentle dissection during which the fiber bundles were separated using sharp forceps. The fibers were then incubated in 3 ml BIOPS buffer containing 50 µg/ml saponin for 30 minutes and subsequently washed in ice-cold buffer (MiR05) for 2 x 10 min. MiR05 contains (mM): EGTA 0.5, $MgCl_2 \cdot 6H_2O$ 3.0, K-lactonionate 60, Taurine 20, KH_2PO_4 10, HEPES 20, Sucrose 110, BSA 1g/l, pH 7.1.

Mitochondrial respiratory measurements

Measurements of oxygen consumption were performed in MiR05 at 37ºC using a polarographic oxygen sensor (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria). Approximately 2.0 to 2.5 mg of muscle tissue (wet weight) was place in either chamber in a cross-sectional design. O_2 flux was resolved by DatLab and all experiments were carried out in hyperoxygenated levels to avoid O_2 diffusion limitations. A sequential substrate addition protocol was used to allow functional dissection of the electron transport system: state 2 respiration (absence of adenylates) was assessed by addition of malate (2 mM) and octanoyl carnitine (1.5 mM), by adding ADP (5 mM) we could reach state 3 respiration for complex I. This was followed by addition of glutamate (10 mM)

and succinate (10 mM) achieving maximal coupled state 3 respiration with parallel electron input to complex I and II. Oligomycin (2 μg/ml) was then added to block complex V and thereafter antimycin A (2.5 μM) to inhibit complex III. Finally ascorbate (2 mM) and TMPD (500 μM) were added to evaluate Complex IV respiration. These mitochondrial respiratory measurements were recorded for both the control group and the experimental group fed the LCHP diet.

Mitochondrial Uncoupling

There were 6 different protocols, some of which were variations of one another, used to test for the presence of uncoupling as we wanted to ensure that the results obtained would not be from a potential uncoupling effect. A portion of the apex tissue mentioned previously was also studied using high-resolution respirometry (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria) to test for uncoupling, running simultaneously along side the mitochondrial respiratory experiments. The first protocol began with the addition of oligomycin (2 μg/ml) followed by succinate (10 mM), then FCCP step titrations (1 μl/step) and finally antimycin A (2.5 μM). The second protocol was the same as the first except GDP (10mM) was added in between the addition of succinate and FCCP. Therefore, uncoupling protein 1 (UCP1)-mediated uncoupling could be studied through the titrations of oligomycin, GDP and antimycin A. The third protocol consisted of the sequential addition of succinate (10 mM), ADP (5 mM) and then glutamate (10 mM) and malate (2 mM) simultaneously. The fourth protocol included the addition of malate (2 mM), octanoyl carnitine (1.5 mM), ADP (5 mM), glutamate (10 mM), oligomycin (2 μg/ml) and then finally multiple additions of GDP (10mM). The fifth protocol began with the addition of malate (2 mM) then octanoyl carnitine (1.5 mM), glutamate (10 mM), oligomycin (2 μg/ml) and again multiple additions of GDP (10mM). The sixth an final protocol consisted of adding malate (2 mM) then octanoyl carnitine (1.5 mM), oligomycin (2 μg/ml) and then multiple additions of GDP (10mM).

Protein Extraction, Immunoblotting, and Immunofluorescence

Tissue from the cross-section excised from right above the apex was pulverized/homogenized using liquid nitrogen with ~150μl lysis buffer containing (in mM) 250 NaCl, 50 HEPES (pH 7.5), 10% glycerol, 1% triton X-100, 1.5 $MgCl_2$ 1 EGTA, 10 $Na_4P_2O_7$ NaF, 800 μM Na_3VO_4. After 1 h on ice the cell slurry was centrifuged at 13,000 rpm for 10 min, and the supernatant was collected. 10μl of lysate was mixed with 2μl DTT and 2μl sample buffer and loaded on a 12.5% acrylamide-SDS gel followed by a transfer onto a 0.45μm nitrocellulose membrane (162-0115 Bio-Rad) in 10mM sodium tetraborate buffer. Ponceau staining was done as loading control before the membranes were blocked in 3% bovine serum albumin (BSA) in 0.1% Tween in Tris-buffered saline (TBS) (10 mm Tris-HCl, pH 7.5, 150 mm NaCl) for 1 hour at room temperature, followed by overnight incubation at 4°C with primary antibody, total OXPHOS rodent antibody cocktail (1:2000, MS604 MitoSciences). The membranes were washed, and incubated with secondary antibody (1:15000, anti-mouse, ab6728 Abcam). Membranes were exposed with ECL chemiluminescence (Immun-Star Chemiluminescent; 1705070; Bio-Rad) and developed bands were analyzed with Image J Software.

Statistical Analysis

Summarized data are presented as means ± standard error of the mean (SEM) for immunoblotting, and standard estimate. Statistical comparisons were done using a two-tailed Student's t-test. n represents the sample number. For all statistical evaluations, In the figures presented, *$p< 0.05$, ** $p< 0.01$.

Results

Relative Expression of Complexes I-V

Immunoblotting revealed an increase in all five complexes in the LCHP diet, normalized to the CON. These trends can be seen in Figure 1 however, only CIV showed a statistically significant difference in expression (CON: 100% ±5.7, LCHP: 122% ±4.8, $p< 0.05$ n= 6). Therefore, the only significant change in

protein expression was in CIV when running samples from CON and LCHP hearts in parallel.

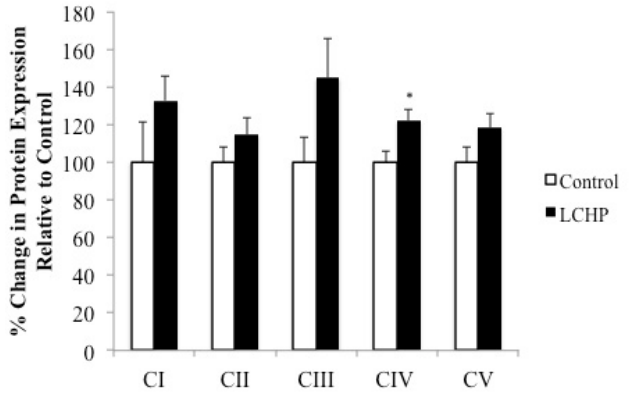

Figure 1: *Immunoblotting of Complex I to Complex V using total OXPHOS rodent antibody cocktail from MitoSciences (MS604). Trends show an increase in all 5 complexes in the LCHP diet relative to the CON diet (CI 100%± 21.4 and 132.7%± 13.1, CII 100%± 8 and 114.8%± 8.5, CIII 100%± 13.3 and 144.9± 20.8, CIV 100%± 5.7 and 122.1%± 5.7 p<0.02, CV 100%± 8.4 and 118.5%± 7.3, respectively, n=6).*

Mitochondrial respiration

We examined oxygen consumption rates of saponin-permeabilized fibres to determine oxidative capacities of mitochondria in the cardiac muscles in situ along with control of the respiratory activity by ADP, the principal regulator. Mitochondrial leak, estimated as antimycin A flux rates subtracted from oligomycin flux rates, indicated a significant increase in the LCHP group as compared to the control group as seen in Figure 3 (52.4 ± 16.1 and 102.2 ± 12.1pmol/s/mg, respectively, p<0.03). There was also a significant difference between these two groups in terms of substrate control ratio for succinate (GM3/GMS3) (0.62 ± 0.08 and 0.44 ± 0.02, respectively, p<0.05) as illustrated in Figure 4. Neither the respiratory control ratio (RCR, state 3 over state 4 respiration so succinate divided by oligomycin respiration) (2.2 ± 0.8 and 3.0 ± 0.6) or the acceptor control ratio (ACR, maximal, ADP stimulated respiration divided by basal, ADP restricted respiration in other words ADP divided by malate respiration) (8.5 ± 1.4 and 12.0 ± 1.4), respectively, a ratio representing the degree of coupling between oxidation and phosphorylation showed any significant differences as seen in Figures 5 and 6. Therefore there was no indication of a likely more efficient oxidation of substrates, nor a significant change within the OXPHOS process. Lipid OXPHOS capacity shown in Figure 7 shows the absence of a significant difference in the lipid coupling control ratio (L/P) between these two same groups (0.33 ± 0.04 and 0.26 ± 0.03). However, there was a significant difference in substrate utilization, as seen in Figure 2. Data show a significant increase in the utilization of octanoyl carnitine (28.3 ± 2.8 and 38.4pmol/s/mg ± 2.9, p<0.03), ADP (75.0 ± 5.6 and 140.0pmol/s/mg ±25.1, p<0.03), glutamate (87.2 ± 5.1 and 161.8pmol/s/mg ± 25.9, p<0.02), succinate (155.5 ± 27.0 and 362.8pmol/s/mg ± 47.9, p<0.01), oligomycin (74.8 ± 14.8 and 122.1pmol/s/mg ± 13.5, p<0.04) and ascorbate+TMPD (212.5 ± 24.8 and 292.9pmol/s/mg ± 19.5, p<0.03) by the LCHP group compared to controls (n=6). Malate and antimycin A utilization were not significant (9.7 ± 1.2 and 11.6pmol/s/mg ± 1.34; 22.4 ± 4.3 and 20.0 pmol/s/mg ± 3.5, respectively). The O_2 flux showed an increased response in the LCHP group as the substrates were titrated as compared to controls (data not shown). Residual oxygen consumption (ROX) was similar in both groups (data not shown).

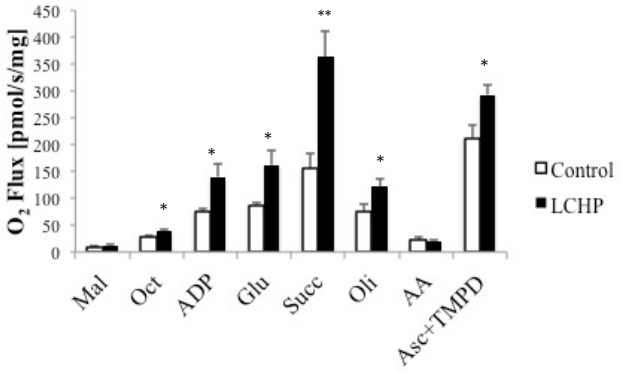

Figure 2*: Substrate utilization by the mitochondria of permeabilized cardiac myofibers assessed through high-resolution respirometry. Data show a significant increase in the utilization of octanoyl carnitine (28.3 ± 2.8 and 38.4pmol/s/mg ± 2.9, p<0.03), ADP (75.0 ± 5.6 and 140.0pmol/s/mg ±25.1, p<0.03), glutamate (87.2 ± 5.1 and 161.8pmol/s/mg ± 25.9, p<0.02), succinate (155.5 ± 27.0 and 362.8pmol/s/mg ± 47.9, p<0.01), oligomycin (74.8 ± 14.8 and 122.1pmol/s/mg ± 13.5, p<0.04) and ascorbate+TMPD (212.5 ± 24.8 and 292.9pmol/s/mg ± 19.5, p<0.03) by the LCHP group compared to controls (n=6). Malate and antimycin A utilization were not significant (9.7 ± 1.2 and 11.6pmol/s/mg ± 1.34; 22.4 ± 4.3 and 20.0 pmol/s/mg ± 3.5, respectively).*

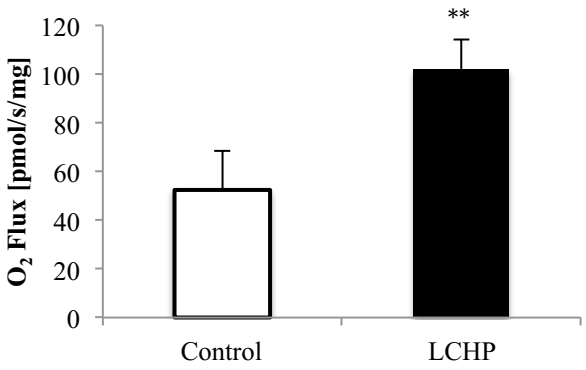

Figure 3*:Mitochodnrial leak assessed by the difference between oligomycin and anitmycin a average rates of respiration. The data reflect a significantly increased mitochondrial leak in the LCHP group compared to controls (102.2 ± 12.1 vs 52.4 ± 16.1pmol/s/mg, respectively, p<0.03, n=6).*

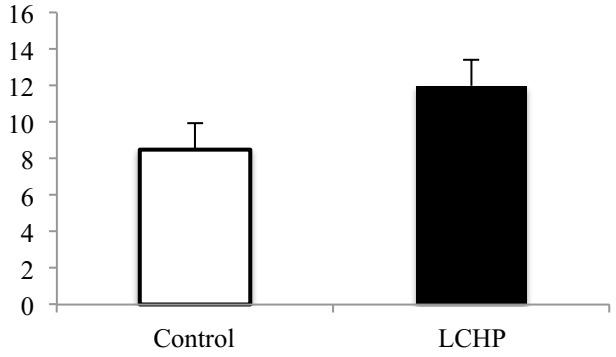

Figure 6*: The acceptor control ratio (ACR) assesses the relative quality of phosphorylation determined by dividing ADP by malate average rates of respiration. The data illustrate a non-significant increase in the ACR of the LCHP group compared to the control group (12.0 ± 1.4 and 8.5 ± 1.4, respectively, n=6).*

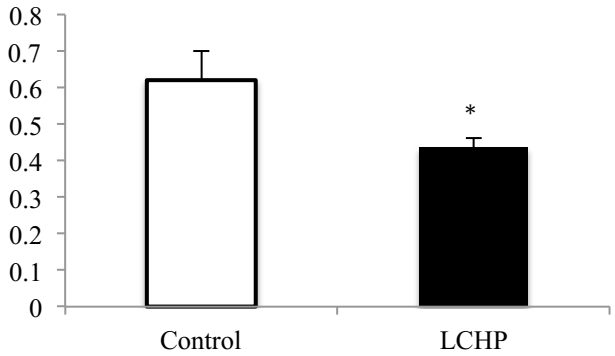

Figure 4*: Substrate control ratio (SCR) representing how the mitochondria handle different substrates expressed by dividing glutamate by succinate average rates of respiration. Data show a significant decrease in this ratio from control to the LCHP group (0.62 ± 0.08 and 0.44 ± 0.02, respectively, p<0.05, n=6).*

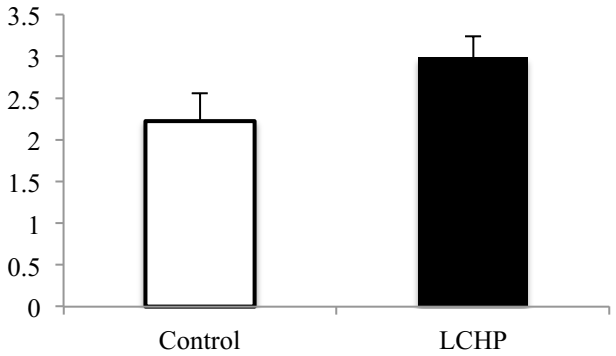

Figure 7*: The respiratory control ratio (RCR) represents state 3 respiration over state 4 respiration obtained by diving succinate by oligomycin average rates of respiration. The data demonstrate a non-significant increase in the RCR of the control group in comparison to the LCHP group (2.2 ± 0.3 and 3.0 ± 0.3, respectively, n=6).*

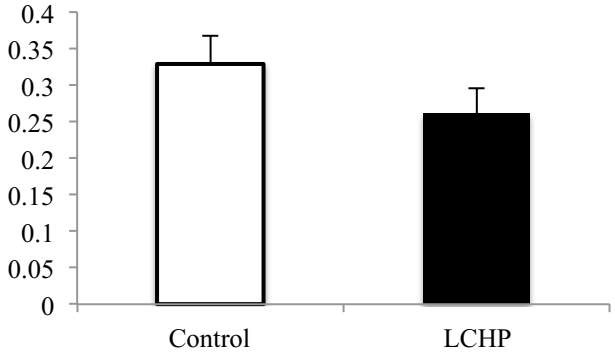

Figure 5*: Lipid coupling control ratio (L/P) reflecting the efficiency to utilize lipids yet under similar respiratory levels, obtained by dividing octanoyl carnitine by glutamate average rates of respiration. The data show a non-significant decrease in the L/P from the control to the LCHP group (0.33 ± 0.04 and 0.26 ± 0.03, respectively, n=6).*

Discussion

The main finding of this study is that animals assigned to a LCHP diet experience a drastic shift in cellular metabolism due to altered macronutrient distribution, favoring an increase in the efficiency of mitochondrial respiration. Taken together, the significant decrease in SCR and increase substrate utilization, specifically of ADP, indicate an increase in the efficiency of the mitochondria to metabolize different substrates. This observation was paralleled by protein expression using immunoblotting, as complexes 1 to 5 were all increased

in the LCHP diet compared to the CON, with a significant increase in complex 4. The relatively high lipid content in the LCHP diet is most probably what impacts the mitochondria in this study rather than the elevated protein content, considering the two main fuel sources for cardiac mitochondria are carbohydrates and lipids. The low-carbohydrate content shifts mitochondrial metabolism in the heart towards its preferred fuel, lipids [53]. Due to the lack of ApoE in the CON and LCHP groups, there is a baseline increase in serum fatty acid levels, which is only supplemented with additional lipid substrate availability in the LCHP group due to the altered macronutrient composition. This may lead to an even more prominent substrate metabolism shift as previously mentioned, that would promote a greater dependency on fatty acids as the source of energy as seen in previous studies [54].

One possible hypothesis to explain our results would be that it may be in fact cytochrome c that is affected by the high lipid environment induced by the LCHP diet and not directly complex IV. The extremely basic and minute hemoprotein, cytochrome c, is plentiful in the mitochondrial intermembrane space. Its main two roles are transporting electrons in mitochondrial respiration and by being released into the cytosol from the mitochondria, it subsequently activates the apoptotic pathway [55-58]. The upregulation of complex IV may be a compensation for how the relatively high lipid content in this diet affects cytochrome c. The second possible hypothesis that could contribute to better interpreting our results would be an upregulation in β-oxidation responding to the elevated fatty acid availability. Complex I and II are less important in creating a proton gradient however, β-oxidation likely provides an extra stimulus somewhere between complexes I or II and complex IV promoting the extra surge of shuttled electrons into complex IV. Cytochrome c's role would also be somewhat implicated in this potential explanation as well.

Fatty acid metabolism has a higher oxygen cost as compared to carbohydrate metabolism which could lead to ischemic-like conditions when the mitochondria experience a prolonged exposure to a high lipid environment. Studies have shown that ischemia leads to a loss of cytochrome c [59]. Therefore in the LCHP group, there may have been a change in the presence of cytochrome c thus in turn affecting complex IV and the electron leak from the cardiac mitochondria, altering overall mitochondrial respiration.

All in all, these speculations are in accordance with our findings since there was an increase in substrate utilization yet a decrease in SCR, implying a decrease in complex I. ADP being significantly increased in the LCHP group along with the significantly increased expression of complex IV could translate into an increased electron flow somewhere in the path towards complex IV, a critical component in removing electrons from the system. This is typically evaluated through effects on cytochrome c.

The significant increase in mitochondrial leak despite no presence of an uncoupling effect, nor a change in the degree of coupling between oxidation and phosphorylation, as seen with the L/P ratio, would have to be further evaluated for thorough understanding of this effect. In addition, variations in mitochondrial density should be assessed with expression of voltage-dependent anion channel (VDAC) using immunoblotting. To further evaluate the hypothetical interpretations of our data, we could monitor changes when using cytochrome c during high-resolution respirometry titrations using the Oxygraph in supplemental experiments, to confirm whether it is indeed affected by this high lipid environment.

References

1. Kopelman PG (2000) Obesity as a medical problem. *Nature.* Vol 6(4): 635-643.

2. Bravata DM, Sanders L, Huang J, Krumholz HM, Olkin I, Gardner CD, Bravata DM (2003) Efficacy and safety of low-carbohydrate diets. A systematic review. *JAMA.* 289(14): 1837-1850.

3. Atkins RC (1998) Dr. Atkins' new diet revolution: New York, NY: Avon Books.

4. Westman EC, Mavropoulos J, Yancy WS, Volek JS (2003) A review of low-carbohydrate ketogenic diets. *Current Atherosclerosis Reports.* 5:476-483.

5. Westman EC, Feinman RD, Mavropoulos JC, Vernon MC, Volek JS, Wortman JA, Yancy WS, Phinney SD (2007) Low-carbohydrate nutrition and metabolism. *Am J Clin Nutr.* 86:276-284

6. Kennedy, ET., Bowman, SA., Spence, JT., Freedman, M. & King, J. (2001). Popular diets: Correlation to health, nutrition, and obesity. *J Am Diet Assoc.* 101:411-420.

7. Krauss, RM., Blanche, PJ., Rawlings, RS., Fernstrom, HS. & Williams, PT. (2006). Separate effects of reduced carbohydrate intake and weight loss on atherogenic dyslipidemia. *Am J Clin Nutr.* 83:1025-1031.

8. Boden G, Sargrad K, Homko C, Mozzoli M, Stein TP (2005) Effect of a low-carbohydrate diet on appetite, blood glucose levels, and insulin resistance in obese patients with type-2 diabetes. *Annals of Internal Medicine.* 142(6): 403-411.

9. Gannon, MC. & Nuttall, FQ. (2003). An increase in dietary protein improves the blood glucose response in persons with type 2 diabetes. *Am J Clin Nutr.* 78: 734-741.

10. Foster, GD., Wyatt, HR., Hill, JO., McGuckin, BG., Brill, C., Mohammed, BS., … Klein, S. (2003). A randomized trial of a low-carbohydrate diet for obesity. *N Engl J Med.* 348: 2082-2090

11. Phillips, SA., Jurva, JW., Syed, AQ., Syed, AQ., Kulinksi, JP., Pleuss, J., … Gutteman, DD. (2008). Benefit of low-fat over low-carbohydrate diet on endothelial healthy in obesity. *Hypertension.* 51: 376-382.

12. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ […] Turner MB (2014) Heart disease and stroke statistics – 2014 update. A report from the American Heart Association. *Circulation.* 128: 1-286.

13. Heart and Stroke Foundation (2013) Heart Failure. Retrieved from http://www.heartandstroke.com/site/c.ikIQLcMWJtE/b.3484065/k.C530/Heart_disease__Heart_failure.htm on March 10, 2015.

14. Tsutsui H, Ide T, Kinugawa S (2006) Mitochondrial oxidative stress, DNA damage, and heart failure. *Antioxidants & Redox Signaling.* Vol 8 (9&10):1737-1744.

15. Belch JJ, Bridges AB, Scott N, Chopra M (1991) Oxygen free radicals and congestive heart failure. *Br Heart J.* 65: 245-248.

16. Hill MF and Singal PK (1996) Antioxidant and oxidative stress changes during heart failure subsequent to myocardial infarction in rats. *Am J Pathol*. 148: 291-300.

17. Hill MF and Singal PK (1997) Right and left myocardial antioxidant responses during heart failure subsequent to myocardial infarction. *Circulation.* 96: 2414-2420.

18. Mallat Z, Philip I, Lebret M, Chatel D, Maclouf J, and Tedgui A. (1998). Elevated levels of 8-iso-prostaglandin F2alpha in pericardial fluid of patients with heart failure: a potential role for in vivo oxidant stress in ventricular dilation and progression to heart failure. *Circulation*. 97: 1536-1539.

19. Ingwall JS, Weiss RG (2004) Is the failing heart energy starved? ON using chemical energy to support cardiac function. *Circ Res.* 95: 135- 145.

20. Neubauer S (2007) The failing heart: an engine out of fuel. *N Eng J Med.* 356: 1140-1151.

21. Stanley WC, Chandler MP (2002) Energy metabolism in the normal and failing heart: potential for therapeutic interventions. *Heart Fail Rev.* 7:115-130.

22. Mettauer B, Zoll J, Garnier A, Ventura-Clapier R (2006) Heart failure: a model of cardiac and skeletal muscle energetic failure. *Pflugers Arch.* 452: 653- 666.

23. Murray AJ, Edwards LM, Clarke K (2007) Mitochondria and heart failure. *Current Opinion in Clinical Nutrition and Metabolic Care.* Vol 10(6): 704-711.

24. Lee L, Campbell R, Scheuermann-Freestone M, et al (2005) Metabolic modulation with perhexiline in chronic heart failure: a randomized, controlled trail of short-term use of a novel treatment. *Circulation.* 112:3280-3288

25. Huss JM, Kelly DP (2005) Mitochondrial energy metabolism in heart failure: a question of balance. *J Clin Invest.* 115: 547-555.

26. Abozguia K, Clarke K, Lee L, Frenneaux M (2006) Modification of myocardial substrate use as a therapy for heart failure. *Nat Clin Pract Cardiovasc Med.* 3: 490-498.

27. Elahi M, Kong Y, Matata B (2009) Oxidative stress as a mediator of cardiovascular disease . Oxid Med Cell Longev;2:259-269.

28. Stanley WC, Recchia FA, Lopaschuk GD (2005) Myocardial substrate metabolism in the normal and failing heart. *Physiol Rev*. 85:1093-1129.

29. Saks V et al. (2006) Molecular system bioenergetics: regulation of substrate supply in response to heart energy demands. *J Physiol.* 577:769-777.

30. Goldberg IJ, Trent CM, Schulze C (2012) Lipid Metabolism and Toxicity in the Heart. *Cell Metabolism*. 15:805-812.

31. Giordano FJ. Oxygen, oxidative stress, hypoxia and heart failure (2005) *J Clin Invest*. 115:500-8.

32. Barth E, Stammler G, Speiser B, Schaper J. (1992) Ultrastructural quantitation of mitochondria and myofilaments in cardiac muscle from 10 different animal species including man. *J Mol Cell Cardiol*. 24:669-681.

33. Bers DM (2001) Excitation-contraction coupling and cardiac contractile force. 2nd edn. Dordrecht/Boston/London:Kluwer Academic Publishers.

34. Lopaschuk GD et al. (1994) Regulation of fatty acid oxidation in the mammalian heart in health and disease. *Biochim Biophys Acta.* 1213:263-276.

35. Akram M (2013) Citric Acid Cycle and Role of its Intermediates in Metabolism. *Cell Biochem Biophys*.1-4.

36. Baldwin JE, Krebs H (1981) The evolution of metabolic cycles. *Nature*. 291:381–2.

37. Bing RJ, Siegel A, Ungar I, Gilbert M. (1954) Metabolism of the human heart II: Studies on fat, ketone and amino acid metabolism. *Am J Med*.16:504-515.

38. Shipp JC, Opie LH, Challoner D. (1961) Fatty acid and glucose metabolism in the perfused heart. *Nature.*189:1018-9.

39. Wisnecki JA, Gertz EQ, Neese RA, Mayr M. (1987) Myocardial metabolism of free fatty acids: studies with ^{14}C labelled substrates in humans. *J Clin Invest.* 79:359-366.

40. Taegtmeyer H (1994) Energy metabolism of the heart: from basic concepts to clinical applications. *Curr Probl Cardiol*. 19:59-113.

41. Fujimoto A, Harary I (1965) The Effects of Lipids on Enzyme Levels in Beating Rat Heart Cells. *Biochem Bioph Res Co*. 20(4): 456-462.

42. Kolwicz SC, Tian R (2011) Glucose metabolism and cardiac hypertrophy. *Cardiovasc Res*. 90:194-201.

43. Mercer JR et al. (2012). The mitochondria-targeted antioxidant MitoQ decreases features of the metabolic syndrome in $ATM^{+/-}$/$ApoE^{-/-}$mice. *Free Radical Biology & Medicine*. 52: 841-849.

44. Clayton DA, Wibom R, Larsson NG (2004) A switch in metabolism precedes increased mitochondrial biogenesis in respiratory chain-deficient mouse hearts. *Proc Natl Acad Sci USA.* 101(9):3136-3141.

45. Sheeran FL, Pepe S. (2006) Energy deficiency in the failing heart: Linking increased reactive oxygen species and disruption of oxidative phosphorylation rate. *Biochim Biophys Acta. 1757*:543-552.

46. Jawien J, NastaNek P & Korbut R. (2004). Mouse models of atherosclerosis. J Physiol Pharmacol 55(3): 503-517.

47. Meir KS & Leitersdorf E. (2004). Atherosclerosis in the apolipoprotein-E-deficient mouse: a decade of progress. Arterioscler Thromb Vasc Biol 24(6): 1006-1014.

48. Meyrelles SS, Peotta VA, Pereira TMC & Vasquez EC. (2011). Endothelial Dysfunction in the Apolipoprotein E-deficient Mouse:insights into the influence of diet, gender and aging. Lipids in Health and Disease 10(211): 1-18.

49. Breslow JL (1993) Trangenic mouse models of lipoprotein metabolism and atherosclerosis. *Proc Natl Acad Sci USA.* 90(18) 8314-8318.

50. Plump AS, Smith JD, Hayek T, Aalto-Setala K, Walsh A, Verstuyft JG, Rubin EM, Breslow JL (1992) Severe hypercholesterolemia and atherosclerosis in apolipoprotein A-deficient mice created by homologous recombination in ES cells. *Cell.* 71(2) 343-353

51. Kuhlencordt PJ, Gyurko R, Han F, Scherrer-Crosbie M, Aretz TH, Hajjar R, Picard MH, Huang PL (2001) Accelerated atherosclerosis, aortic aneurysm formation, and ischemic heart disease in apolipoprotein A/endothelial nitric oxide synthase double-knockout mice. *Circulation.* 104(4): 448-454.

52. Zhang SH, Reddick RL, Piedrahita JA, Maeda N (1992) Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science.* 258(5081): 468-471.

53. Berg JM, Tymoczko JL, Stryer L (2002) Biochemistry, 5th Ed. New York: WH Freeman.

54. Rocha C, Scheede-Bergdahl C, Whitesell T & Bergdahl A. (2014) Implications of Apolipoprotein E Deficiency on Cardiac Mitochondrial Oxygen Consumption in a

Young Mouse Model. *European Journal of Cardiovascular Medicine*. 3(1): 394-400.

55. Trusova, VM, Gorbenko GP, Molotkovsky JG & Kinnunen PKJ. (2010) Cytochrome c-Lipid Interactions: New Insights from Resonance Energy Transfer. *Biophysical Journal*. 99(6): 1754–1763.

56. Pinheiro TJT, et al. (2003) Direct evidence for the cooperative unfolding of cytochrome c in lipid membranes from H-2 H exchange kinetics. *J. Mol. Biol.* 303(2): 617–626.

57. Shidoji, YK, et al. (1999) Loss of molecular interaction between cytochrome c and cardiolipin due to lipid peroxidation. *Biochem. Biophys. Res. Commun,* 264(3): 343–347.

58. Schug, ZT, & Gottlieb, E. (2009) Cardiolipin acts as a mitochondrial signalling platform to launch apoptosis. *Biochim. Biophys. Acta*. 1788:2022–2031.

59. Borutaite V, Toleikis A & Brown GC. (2013) In the eye of the storm: mitochondrial damage during heart and brain ischaemia. *FEBS Journal.* 280: 4999–5014.

V

Concluding Remarks

Concluding Remarks

The goal of this thesis was to better understand the role diet plays in terms of cardiovascular disease. In particular, my focus was on low-carbohydrate high-protein diets and their safety or risks involved. These diets contain negligible amounts of carbohydrates and high amounts of protein and fat. The lack of carbohydrates induces a shift in metabolism towards more fat oxidation rather than glucose utilization. This shift does not happen without consequence, and data from my project demonstrate that cellular and biochemical mechanisms are indeed being affected. Vascular smooth muscle cells demonstrated a loss in contractile proteins supporting the notion that the cells are undergoing a phenotype shift from contractile to synthetic, however this effect was not translated to a compromised functional capacity. This led us to believe that patients more susceptible to endothelial damage, with history of vessel occlusion, or stent implantation should practice a certain degree of caution when engaging on low-carbohydrate diets. However, in the project assessing mitochondrial function, we saw a beneficial effect induced by the use of fatty acids and a lipid-rich environment. The cardiac mitochondria prefer lipid to produce energy and demonstrated a favorable outcome, utilizing substrates and lipids more efficiently using lipids for ATP production.

To further expand this project, the following suggestions are recommended: measure additional contractile proteins with immunoblotting for dedifferentiation evaluation, incorporate another experimental group 12 weeks of age to re-assess functional capacity by wire myography and mitochondrial functioning for an aging effect, include cytochrome c to analyze electron flow in cardiac mitochondria, and lastly measure reversibility of damage by adding and removing the diet for equal amounts of time. This will bring vital information for both clinicians and patients

alike to make a more thorough assessment of the safety or risks associated with these diets on our cardiovascular system.

VI

References

References

1. Statistics Canada. (2012). Leading Causes of Death in Canada.

2. Statistics Canada. (2011). Mortality, summary list of causes 2008.

3. Davies, MG. & Hagen, PO. (1994). Pathobiology of intimal hyperplasia. *Br J Surg.* 81: 1254-1269.

4. Watanabe, T., Haraoka, S., Shimokama, T. (1996). Inflammatory and immunological nature of atherosclerosis. Int J Cardiol; 54 (suppl): S51-60.

5. Jonasson, L., Holm, J., Skalli, O., Bondjers, G., Hansson, GK. (1986). Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. Arteriosclerosis; 6: 131-138.

6. Mendis, S., Puska, P., Norrving, B., editors. (2011). Geneva: World Health Organization; 2011. Global Atlas on Cardiovascular Disease Prevention and Control.

7. Promocell (n.d.). Endothelial Cells (Large Vessels). *Promocell.* Retrieved March 6, 2014, from http://www.promocell.com/products/human-primary-cells/endothelial-cells-large-vessels/

8. Cipollone, F., Fazia, ML. & Mezzetti, A. (2007). Oxidative stress, inflammation, and atherosclerotic plaque development. *International Congress Series.* 1303: 35-40.

9. Gonzalez, AM. & Selwyn, AP. (2003). Endothelial function, inflammation, and prognosis in cardiovascular disease. *Am J Med.* 115 (8A): 99S-106S.

10. Ross, R. (1993). The pathogenesis of atherosclerosis. *Nature.* 362; 801-809.

11. Saini, HK. (2006). Modification of biochemical and cellular processes. In SK Cheema (Ed.), Biochemistry of Atherosclerosis (pp. 473-494). New York, NY: Springer.

12. Ross, R., Glomset, J., Harker, L. (1977). Response to injury and atherogenesis. *Am J Pathol.* 86: 675-684.

13. Berman, JW., Kazimi, M., Ma, H. (2002). Development of the Atherosclerotic Plaque. In D.L. Brown (Ed.), *Cardiovascular Plaque Rupture* (pp. 1-50). New York, NY: Marcel Dekker.

14. Hansson GK (2005) Mechanisms of disease- inflammation, atherosclerosis, and coronary artery disease. *NEJM.* Vol 352(16) 1685-1695.

15. Skalen, K., Gustafsson, M., Rydberg, EK., et al. (2002). Subendothelial retention of Atherogenic lipoproteins in early atherosclerosis. *Nature.* 417: 750-754.

16. Leitinger, N. (2002). Oxidized phospholipids as modulators of inflammation in atherosclerosis. *Curr Opin Lipidol.* 14: 421-430.

17. Dai, G., Kaazempur-Mofrad, MR., Natarajan, S, et al. (2004). Distinct endothelial phenotypes evoked by arterial waveforms derived from atherosclerosis-susceptible and –resistant regions of human vasculature. *Proc Natl Acad Sci USA.* 101: 14871-14876.

18. Massberg, S., Brand, K., Gruner, S. et al. (2002). A critical role of platelet adhesion in the initiation of atherosclerotic lesion formation. *J Exp Med.* 196: 887-896.

19. Eriksson, EE., Xie, X., Werr, J., Thoren, P. & Lindbom, L. (2001). Importance of primary capture and L-selectin-dependent secondary capture in leukocyte accumulation in inflammation and atherosclerosis in vivo. *J Exp Med.* 194: 205-218.

20. Cybulsky, MI. & Gimbrone, MA Jr. (1991). Endothelial expression of a mononuclear leukocyte adhesion during atherogenesis. *Science.* 251: 788-791.

21. Peiser, L., Mukhopadhyay, S. & Gordon, S. (2002). Scavenger receptors in innate immunity. *Curr Opin Immunol.* 14: 123-128.

22. Janeway, CA Jr. & Medzhitov, R. (2002). Innate immune recognition. *Annu Rev Immunol.* 20: 197-216.

23. Cullen, P., Rauterberg, J., Lorkowski, S. (2005). The Pathogenesis of Atherosclerosis. In A. Von Eckardstein (Ed.), *Atherosclerosis: Diet and Drugs* (pp. 3-70). New York, NY: Springer.

24. Braganza, DM & Bennett, MR. (2001). New insights into atherosclerotic plaque rupture. *Postgrad Med J.* 77: 94-98.

25. Kovanen, PT., Kaartinen, M. & Paavonen, T. (1995). Infiltrates of activated mast cells at the site of coronary atheromatous erosion or rupture in myocardial infarction. *Circulation.* 92:1084-1088.

26. Van der Wal, AC., Becker, AE., Van der Loos., CM. & Das, PK. (1994). Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. *Circulation.* 89:36-44.

27. Moreno, PR., Falk, E., Palacios, IF., Newell, JB., Fuster, V. & Fallon, JT. (1994). Macrophage infiltration in acute coronary syndromes: implications for plaque rupture. *Circulation.* 90:775-778.

28. Hansson, GK., Hellstrand, M., Rymo, L., Rubbia, L. & Gabbiani, G. (1989). Interferon-gamma inhibits both proliferation and expression of differentiation-specific alpha-smooth muscle cells. *J Exp Med.* 170: 1595-1608.

29. Amento, EP, Ehsani, N., Palmer, H. & Libby, P. (1991). Cytokines and growth factors positively and negatively regulate interstitial collagen gene expression in human vascular smooth muscle cells. *Arterioscler Thromb.* 11: 1223-1230.

30. Saren, P., Welgus, HG. & Kovanen, PT. (1996). TNF-alpha and IL-1beta selectively induce expresson of 92-kDa gelatinase by human macrophages. *J Immunol.* 157:4159-4165.

31. Mach, F., Schonbeck, U., Bonnefoy, JY., Pober, JS. & Libby, P. (1997). Activation of monocyte/macrophage functions related to acute atheroma complication by ligation of CD40: induction of collagenase, stromelysin, and tissue factor. *Circulation.* 96:396-399.

32. Schloen, FJ. (2005). Blood vessels. In: Kumar, V., Abbas, AK., Kausto, N (Eds.). Robbins and Cotran Pathologic Basis of Disease (pp. 525). 7th ed. Philedelphia, PA: Elsevier Saunders.

33. Libby, P., Ridker, PM. & Maseri, A. (2002). Inflammation and atherosclerosis. *Circulation*. 105: 1135-1143

34. Danesh, J., Collins, R., Peto, R. (1997). Chronic infections and coronary heart disease: is there a link? *Lancet.* 350: 430-436.

35. Saikku, P., Leinonen, M., Mattila, K, et al. (1988). Serological evidence of an association of a novel chlamydia, TWAR, with chronic coronary heart disease and acute myocardial infarction. *Lancet*. 2: 93-986.

36. Sherer Y and Shoenfeld Y (2006) Mechanisms of disease: atherosclerosis in autoimmune disease. *Nature Clinical Practice Rheumatology.* Vol 2(2): 99-106.

37. George J, Greenberg S, Barshack I, et al. (2001) Accelerated intimal thickening in carotid arteries of balloon injured rats after immunization against heat shock protein 70. *J Am Coll Cardiol.* Vol38(5): 1564-1569.

38. Xu QB, Willeit J, Marosi M, et al. (1993) Association of serum antibodies to heat-shock protein-65 with carotid atherosclerosis. *Lancet.* Vol 341(8840): 255-259.

39. Owens, GK., Kumar, MS. & Wamhoff, BR. (2004). Molecular regulation of vascular smooth muscle cell dedifferentiation in development and disease. *Physio Rev.* 84: 767-801.

40. Chan, Philip. (2007). Vascular smooth muscle cells: structure and function. In R. Fitridge & M. Thompson (Eds.), *Mechanisms of Vascular Disease: a textbook for vascular surgeons* (pp. 14-21). New York, NY: Cambridge University Press.

41. Campbell, G & Campbell G. (1990). The phenotypes of smooth muscle expressed in human atheroma. *Ann NY Acad Sci.* 598: 143-158.

42. Bochaton-Piallat, M-L. & Gabbiani, G. (2005). Modulation of Smooth Muscle Cell Proliferation and Migration: Role of Smooth Muscle Cell Heterogeneity. In A. Von Eckardstein (Ed.), *Atherosclerosis: Diet and Drugs* (pp. 645-663). New York, NY: Springer.

43. Milewicz, DM., Kwartler, CS., Papke, CL., Regalado, ES., Cao, J. & Reid, AJ. (2010). Genetic variants promoting smooth muscle cell proliferation can result in diffuse and diverse vascular diseases: Evidence for a hyperplastic vasculopathy. *Genetics in Medicine.* 12:196-203.

44. Kocher, O., Skalli, O., Bloom, WS., Gabbiani, G. (1984). Cytoskeleton of rat aortic smooth muscle cells. Normal conditions and experimental intimal thickening. *Lab Invest.* 50: 645-652.

45. Gabbiani, G., Rungger-Brandle, E., de Castonay, C., Franke, WW. (1982). Vimentin-containing smooth muscle cells in aortic intimal thickening after endothelial injury. *Lab Invest.* 47: 265-269.

46. Kocher, O., Gabbiani, F., Gabbiani, G., et al. (1991). Phenotypic features of smooth muscle cells during the evolution of experimental carotid artery intimal thickening. Biochemical and morphologic studies. *Lab Invest.* 65: 459-170.

47. Owens, GK. (1995). Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev.* 75: 487-517.

48. Clowes, AW., Reidy, MA., Clowes, MM. (1983). Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium. *Lab Invest.* 49: 327- 333.

49. Dzau, VJ., Braun-Dullaeus, RC., Sedding, DC. (2002). Vascular proliferation and atherosclerosis: new perspectives and therapeutic strategies. *Nature Medicine.* 8: 1249-1256.

50. Klagsbrun, M. & Edelman, ER. (1989). Biological and biochemical properties of fibroblast growth factors. Implications for the pathogenesis of atherosclerosis. *Arteriosclerosis.* 9: 269-278.

51. Bjornsson, TD., Dryjski, M., Tluczek, J., et al. (1991). Acidic fibroblast growth factor promotes vascular repair. *Proc Natl Acad Sci USA.* 88: 8651-8655.

52. Sara, VR. & Hall, K. (1990). Insulin-like growth factors and their binding proteins. *Physiol Rev.* 70: 591-614.

53. Okazaki, H., Majesky, MW., Harker, LA., Schwartz, SM. (1992). Regulation of platelet-derived growth factor ligand and receptor gene expression by alpha-thrombin in vascular smooth muscle cells. *Circ Res.* 71: 1285-1293.

54. Davies, MG. & Hagen, PO. (1994). Pathobiology of intimal hyperplasia. *Br J Surg.* 81: 1254-1269.

55. Rogers, C., Edelman, ER., Simon, DI. (1998). A mAb to the beta2-leukocyte integrin Mac-1 (CD11b/CD18) reduces intimal thickening after angioplasty or stent implantation in rabbits. *Proc Natl Acad Sci USA.* 95:10134-10139.

56. Becker, AE. & van der Wal, AC. (2002). The Role of Inflammation in Plaque Rupture. In D.L. Brown (Ed.), *Cardiovascular Plaque Rupture* (pp. 63-78). New York, NY: Marcel Dekker.

57. McCardle, WD., Katch, FI. & Katch, VL. (2010). Nutrition: The base for human performance. In McCardle, WD., Katch, FI. & Katch, VL (Eds.), *Exercise Physiology; Nutrition, Energy and Human Performance* (7th ed.) Baltimore, MD: Lipincott Williams and Wilkins.

58. Adam-Perrot, A., Clifton, P. & Brouns, F. (2006). Low-carbohydrate diets: nutritional and physiological aspects. *Obesity Review.* **7,** 49-58.

59. Westman EC, Mavropoulos J, Yancy WS, Volek JS (2003) A review of low-carbohydrate ketogenic diets. *Current Atherosclerosis Reports.* 5:476-483.

60. Bartlett, K. & Eaton, S. (2004). Mitochondrial β-oxidation. *Eur J Biochem.* 271:462-469.

61. Jeukendrup, A. & Gleeson, M. (2010). Fuel sources for muscle and exercise metabolism. In Jeukendrup, A. & Gleeson, M (Eds.), *Sport Nutrition* (2nd ed) Champaign, IL: Human Kinetics.

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