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# Food Restriction and Atherosclerotic Plaque Stabilization

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Dorien G. De Munck, Guido R.Y. De Meyer and Wim Martinet

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## Abstract

Food restriction is a promising therapy for many age-associated pathologies as it stimulates the health-supportive mechanism autophagy. Because atherosclerosis is an inflammatory, age-related disease, dietary modification can be an important strategy in preventing atherosclerotic plaque development. A cholesterol-supplemented diet, used to induce plaque formation in rabbits, induces a pronounced hypercholesterolemia, which can be reversed after 4 weeks of normal diet. However, food restriction induces a further increase in circulating LDL cholesterol. These elevated cholesterol levels are associated with the induction of autophagy. Although neither a short-term normal diet nor food restriction alters plaque size, rabbits fed a normal diet show signs of increased plaque stability such as elevated collagen content and decreased expression of vascular cell adhesion molecule (VCAM)-1. Surprisingly, these favorable effects are not present after 4 weeks of food restriction. On the contrary, atherosclerotic plaques of food-restricted rabbits displayed enhanced apoptosis, a process known to further undermine plaque stability. In conclusion, severe short-term food restriction in rabbits prevents stabilization of atherosclerotic plaques as observed after regular cholesterol withdrawal via a normal diet.

**Keywords:** atherosclerosis, plaque stability, food restriction, cholesterol, autophagy

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## 1. Introduction

Atherosclerosis is an inflammatory disease characterized by the formation of plaques in the large- and medium-sized arteries. Despite current pharmacological therapies, atherosclerosis remains the leading cause of death and morbidity among adults in the Western world [1]. Because a diet rich in calories, together with a sedentary lifestyle, contributes to the development

of atherosclerosis, dietary change is considered an important strategy in the prevention of atherosclerosis [2]. Moreover, dietary modification has shown to play an important role in several age-associated pathologies and in aging itself. Moderate calorie restriction results in a lifespan expansion of different species including yeast, fruit flies, nematodes, fish, rodents, and rhesus monkeys [3]. Besides favorable effects on longevity, long-term as well as short-term caloric restriction improves the cardiovascular disease risk profile in humans [4, 5]. Consistent with this finding, animal studies showed that dietary restriction attenuates atherosclerotic plaque development and decreases endothelial dysfunction [6, 7].

Starvation, as an extreme form of food restriction, is also one of the most important stimuli for autophagy induction [8]. Autophagy is a subcellular degradation pathway for long-lived proteins and damaged organelles. Under normal conditions, autophagy is a homeostatic process that is found in all cell types. However, under stress conditions, it functions as an important cell survival mechanism through nutrient recycling and the generation of energy [9]. Growing evidence indicates that autophagy deficiency plays a crucial role in plaque growth and destabilization [10–12]. Moreover, autophagy induction is suggested as a novel strategy for the prevention and treatment of atherosclerosis [13, 14].

## 2. Food restriction induces hypercholesterolemia

Cholesterol withdrawal by feeding atherosclerotic rabbits a normal diet for 4 weeks significantly reduces LDL cholesterol in serum (**Table 1**). In contrast, cholesterol withdrawal by severe food restriction (only 20% of normal diet) leads to elevated LDL cholesterol levels and a significant loss of bodyweight (**Table 1**), which confirms previous studies showing hypercholesterolemia in healthy subjects after fasting or moderate caloric restriction [15–17] as well as in patients with eating disorders such as anorexia nervosa [18]. Several mechanisms may account for hypercholesterolemia including downregulation of the hepatic LDL receptor leading to decreased LDL uptake in the liver, lipolysis in adipose tissue, or increased cholesterol synthesis [15, 17, 19].

	Weeks	Baseline	Normal diet	Restricted diet
LDL cholesterol (mg/dL)	20	1026 ± 147	589 ± 98	702 ± 13
	24	/	250 ± 105 <sup>##</sup>	1101 ± 177 <sup>#,***</sup>
Triglycerides (mg/dL)	20	92 ± 29	53 ± 13	56 ± 9
	24	/	49 ± 7	44 ± 9
Bodyweight (kg)	20	4.0 ± 0.2	4.3 ± 0.1	4.0 ± 0.1
	24	/	4.4 ± 0.1	3.2 ± 0.1 <sup>###,***</sup>

Data are expressed as mean ± SEM.

<sup>#</sup>P < 0.05.

<sup>##</sup>P < 0.01.

<sup>###</sup>P < 0.001 versus 20 weeks (paired sample t-test, n = 10).

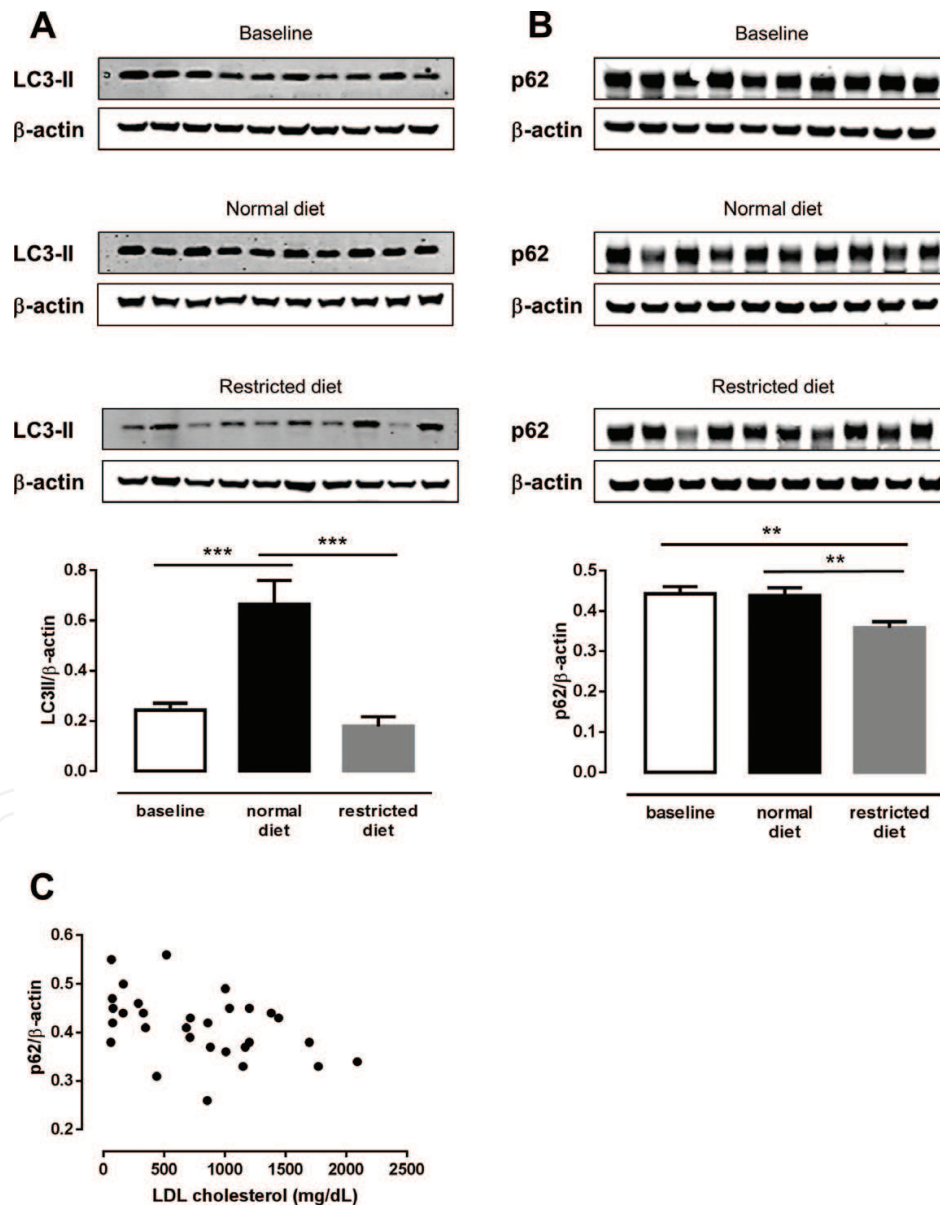
<sup>\*\*\*</sup>P < 0.001 versus normal diet (independent sample t-test, n = 10).

**Table 1.** Serum lipid values and body weight in cholesterol-fed rabbits (baseline, 20 weeks of cholesterol) followed by dietary lipid lowering for 4 weeks (normal diet) or a restricted diet for 4 weeks (restricted diet).

Despite increased levels of circulating LDL, there is no difference in lipid accumulation in the liver or aorta of rabbits undergoing severe food restriction. Both normal diet and food restriction do not affect serum triglycerides (Table 1).

### 3. Hypercholesterolemia induced by food restriction is associated with autophagy induction

LDL cholesterol levels are negatively correlated with SQSTM1/p62 protein levels in the liver (Figure 1), suggesting stimulation of autophagy as an alternative mechanism for the increase



**Figure 1.** Induction of autophagy in liver of rabbits that were fed 0.3% cholesterol for 20 weeks (baseline), followed by cholesterol withdrawal for 4 weeks either via a normal diet or a restricted diet (20% of normal diet). Liver samples of ten rabbits per group were analyzed by Western blotting for the expression of autophagy marker proteins LC3-II (A) and p62 (B). \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (One-way ANOVA with post-hoc LSD,  $n = 10$  in each group). (C) Serum LDL-levels show an inverse correlation with liver p62 protein levels (Pearson Correlation Coefficient  $-0.44$ ,  $P < 0.05$ ).

in serum lipids. SQSTM1/p62 is a scaffold protein that binds directly to the autophagosomal marker Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates via autophagy. Nutrient deprivation is a powerful autophagy-inducing condition [20]. Rabbits that undergo cholesterol withdrawal via a normal diet show high LC3-II levels but unaltered amounts of SQSTM1/p62 (**Figure 1A**), indicating moderate induction of autophagy. In contrast, rabbits undergoing severe food restriction show low levels of LC3-II and a clear reduction of SQSTM1/p62 (**Figure 1B**), which points to strong autophagy stimulation. It has been described that autophagy is strongly involved in managing intracellular lipids [21, 22]. Lipid droplets are taken up by lysosomes, where lysosomal acid lipases hydrolyze cholesteryl esters to generate free cholesterol for ATP-binding cassette transporter 1 (ABCA1)-mediated cholesterol efflux [21]. Impairment of autophagy in macrophages reduces reverse cholesterol transport [21], a condition that refers to net cholesterol flux from the peripheral tissues to the liver (for excretion via the bile). Conversely, pharmacological activation of the autophagy pathway attenuates lipid accumulation [23] and in some conditions (e.g., after treatment with mTOR inhibitors) triggers hypercholesterolemia [24].

#### 4. Cholesterol withdrawal increases plaque stability

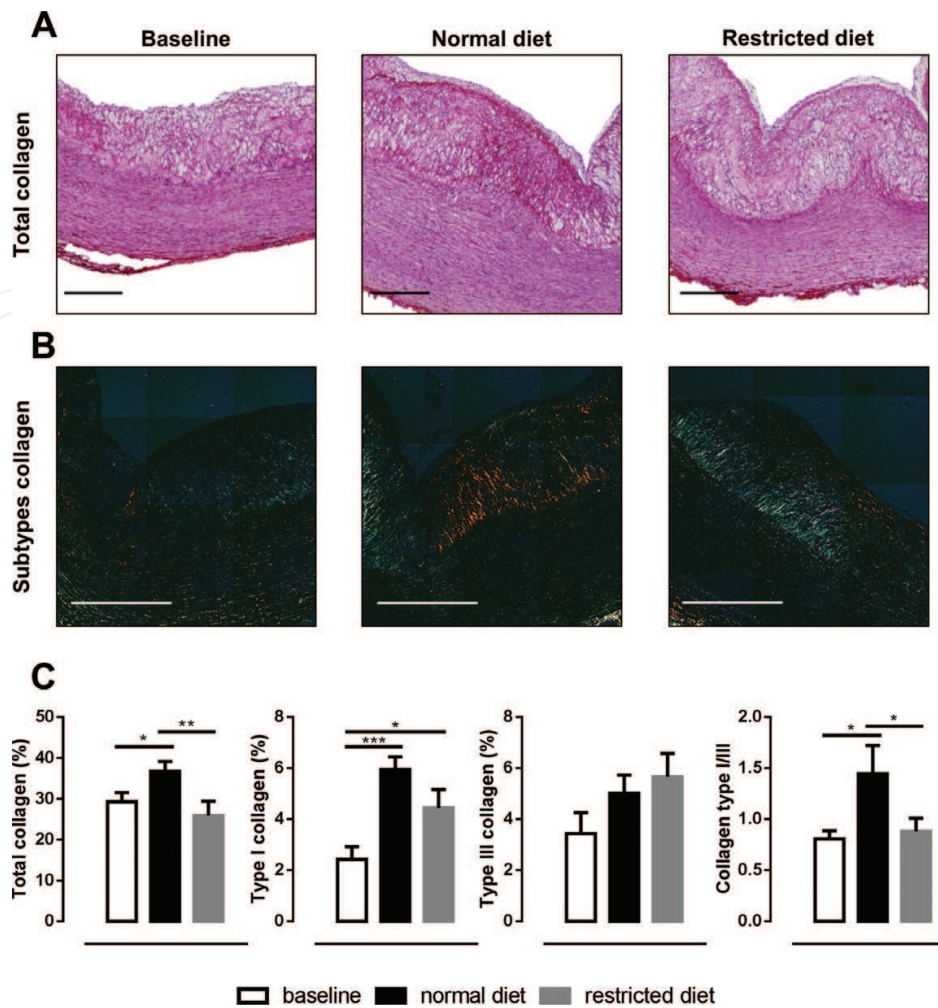
Numerous studies indicate that dietary modification is an important strategy for the prevention of cardiovascular disease [2, 25]. However, studies examining the effects of food restriction on atherosclerosis are scarce. Although short-term cholesterol withdrawal (4 weeks) does not alter plaque size (**Table 2**), a normal unrestricted diet results in a more stable plaque phenotype. Indeed, collagen content of the atherosclerotic plaques is increased, mainly due to an increase in type I collagen (**Figure 2**), which is essential for plaque stability. Moreover, VCAM-1 expression in endothelial cells declines (**Figure 3**). VCAM-1 is important for leucocyte recruitment and thereby contributes to plaque inflammation and macrophage accumulation. However, despite a decrease in VCAM-1 expression, the total amount of macrophages in the plaque does not alter within 4 weeks of cholesterol withdrawal (4 weeks). Indeed, only prolonged cholesterol withdrawal (12–24 weeks) results in a dramatic loss of plaque macrophages [26–28].

	Baseline	Normal diet	Restricted diet
Plaque area (mm <sup>2</sup> )	3.3 ± 0.8	3.6 ± 0.6	3.6 ± 1.0
Macrophages (%)	22 ± 3	24 ± 4	34 ± 6
Smooth muscle cells (%)	26 ± 4	23 ± 2	26 ± 4
Fibrous cap thickness	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1

Data are expressed as mean ± SEM.

**Table 2.** Plaque area and cellular composition in the proximal ascending in cholesterol-fed rabbits (baseline, 20 weeks of cholesterol) followed by dietary lipid lowering for 4 weeks (normal diet) or a restricted diet for 4 weeks (restricted diet).



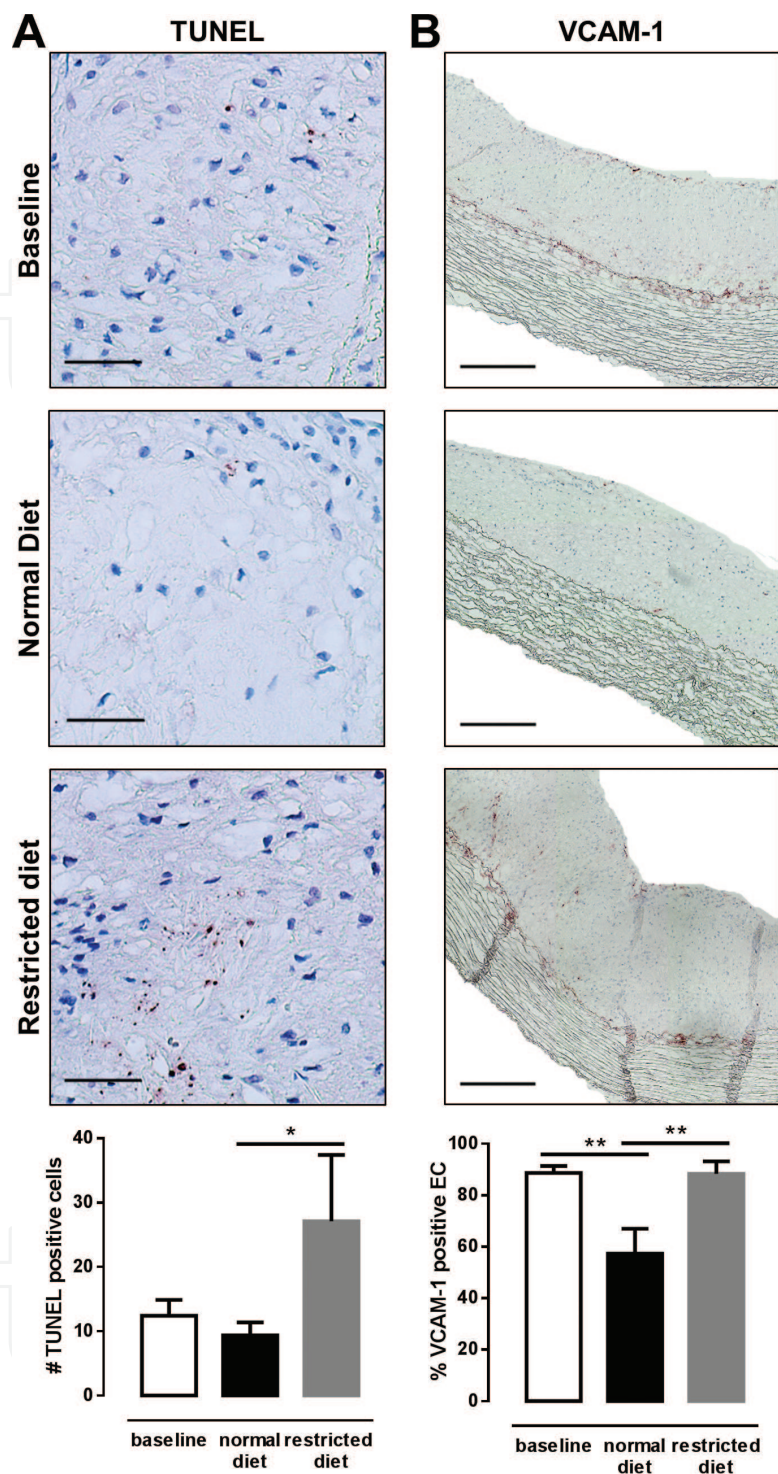


**Figure 2.** Collagen content of atherosclerotic plaques in rabbits that were fed 0.3% cholesterol for 20 weeks (baseline), followed by cholesterol withdrawal for 4 weeks either via a normal diet or a restricted diet (20% of normal diet). (A) Sections of the proximal ascending aorta were stained with Sirius red for total collagen determination. Scale bar = 500  $\mu$ m. (B) Analysis of Sirius red staining via polarized light microscopy. Collagen type I is displayed in red, type III in green. Scale bar = 500  $\mu$ m. (C) Quantification of total collagen, type I and type III collagen as well as the type I/III collagen ratio in Sirius red stained sections. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (One-way ANOVA with post-hoc LSD,  $n = 8-10$  in each group).

## 5. Effect of food restriction on plaque development is controversial

In contrast with a normal unrestricted diet, severe food restriction does not promote beneficial effects such as increased collagen synthesis and decreased VCAM-1 expression. On the contrary, plaques of rabbits undergoing food restriction reveal an increase in apoptosis (Figure 3). Depending on the cell type and stage of the plaque, apoptosis could be detrimental for plaque stability [29]. Moreover, apoptosis can stimulate the release of inflammatory cytokines and chemotactic factors, thereby further aggravating plaque inflammation [30].

Given that food restriction stimulates autophagy, a well-known cellular survival mechanism, increased apoptosis may seem surprising. However, autophagy induction after intensive nutrient deprivation may be insufficient to counteract apoptosis.



**Figure 3.** Atherosclerotic plaque composition of rabbits that were fed 0.3% cholesterol for 20 weeks (baseline), followed by cholesterol withdrawal for 4 weeks either via a normal diet or a restricted diet (20% of normal diet). (A) Sections of the proximal ascending aorta were TUNEL stained for the detection of apoptosis and the number of TUNEL positive cells in each group was quantified. Scale bar = 50  $\mu$ m. \* $P < 0.05$  (One-way ANOVA with post-hoc LSD,  $n = 10$  in each group). (B) Sections of the proximal ascending aorta were immunohistochemically stained for VCAM-1 expression on endothelial cells. The number of VCAM-1 positive endothelial cells in each group was quantified. Scale bar = 500  $\mu$ m. \*\* $P < 0.01$  (One-way ANOVA with post-hoc LSD,  $n = 8$ –10 in each group).

The abovementioned findings are in agreement with previous studies in rabbits showing increased plaque development after a 50% reduction in food intake [31], even though Lacombe et al. [16] reported that aggravated atherosclerosis only occurs in rabbits when dietary restriction is combined with cholesterol feeding. Prenatal under nutrition is also known to program a pro-atherosclerotic phenotype and to accelerate plaque development in young adult offspring [32, 33]. Nonetheless, a large body of evidence indicates that food restriction is associated with a range of positive effects on cardiovascular health. Dietary restriction in apolipoprotein E-deficient mice, for example, results in the development of smaller and less advanced atherosclerotic lesions [7, 34]. A lower incidence of atherosclerotic plaque development is also seen in genetically obese rats consuming a low calorie diet, as compared to rats fed ad libitum [35]. Studies in humans clearly describe a reduction in cardiovascular risk factors but often fail to demonstrate a direct effect on atherosclerotic plaque development [4, 5]. Still, the incidence of atherosclerosis was decreased during the years following World War I and World War II, which supports the general benefit of food deprivation [36]. Importantly, at least two main differences in the experimental design or setup of different studies should be mentioned that may explain a different outcome. First, differences might be related to the severity of food restriction (50% food restriction = moderate, 80% food restriction = severe). Accordingly, severe food restriction holds a higher risk of vitamin deficiency that should be taken into account. Indeed, vitamin D deficiency may contribute to atherosclerosis [37], and also vitamin C and vitamin E depletions are demonstrated to aggravate plaque development [38]. Second, the time span of dietary restriction could be an important factor. Four weeks of food restriction is relatively short in comparison with other studies showing beneficial effects of food restriction. Fontana et al. [4], for example, reported a reduced risk for atherosclerosis in individuals who had been on food restriction for an average of 6 years.

In conclusion, severe short-term food restriction seems to counteract the plaque stabilizing benefits of cholesterol withdrawal in rabbits.

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## Author details

Dorien G. De Munck, Guido R.Y. De Meyer and Wim Martinet\*

\*Address all correspondence to: [wim.martinet@uantwerpen.be](mailto:wim.martinet@uantwerpen.be)

Laboratory of Physiopharmacology, University of Antwerp, Antwerp, Belgium



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