

## Original Article

# Hypoascorbemia induces atherosclerosis and vascular deposition of lipoprotein(a) in transgenic mice

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**Abstract:** Lipoprotein(a), a variant of LDL carrying the adhesive glycoprotein apo(a), is a leading risk factor for cardiovascular disease. Lipoprotein(a) (Lp(a)) is found in humans and subhuman primates but rarely in lower mammals. Better understanding of the evolutionary advantage of this molecule should elucidate its physiological role. We developed a new mouse model with two characteristics of human metabolism: the expression of Lp(a) and the lack of endogenous ascorbate (vitamin C) production. We show that dietary deficiency of ascorbate increases serum levels of Lp(a). Moreover, chronic hypoascorbemia and complete depletion of ascorbate (scurvy) leads to Lp(a) accumulation in the vascular wall and parallels atherosclerotic lesion development. The results suggest that dietary ascorbate deficiency is a risk factor for atherosclerosis independent of dietary lipids. We provide support for the concept that Lp(a) functions as a mobile repair molecule compensating for the structural impairment of the vascular wall, a morphological hallmark of hypoascorbemia and scurvy.

**Keywords:** Lipoprotein(a), Lp(a), vitamin C, hypoascorbemia, atherosclerosis, vascular plaques, *Gulo*<sup>-/-</sup>, Lp(a) transgene, cvd risk factors

## Introduction

Lipoprotein(a) (Lp(a)) is one of the strongest genetic risk factors for cardiovascular disease (CVD), including myocardial infarctions, stroke and other forms of CVD [1, 2]. Lipoprotein(a) is composed of a low-density lipoprotein (LDL) molecule and a high molecular weight glycoprotein, apolipoprotein(a) (apo(a)), bound to the primary structural protein of LDL, apolipoprotein B-100 (apoB-100), via disulfide bonds [3].

Lipoprotein(a) has been shown to accumulate within human atherosclerotic plaques [4] and to bind to components of the extracellular cellular matrix (ECM), including fibronectin, laminin, and others [5]. Moreover, apo(a) is known to enhance smooth muscle cell replication [6]. Due to its homology with plasminogen [7], apo(a) binds avidly to fibrinogen and fibrin, displaying strong antifibrinolytic properties. Thus, Lp(a) has been described as the “missing link” between atherogenesis and thrombogenesis [8]. While many characteristics of this molecule have been elucidated since its discovery [9] half a century ago, its physiological significance is still poorly understood [10].

Lipoprotein(a) is primarily found in humans and sub-human primates and the emergence of the *apo(a)* gene was dated to about 40 million years ago, about the time of the divergence of the Old World and New World monkeys [7]. This date coincides with the loss of endogenous ascorbate (vitamin C) synthesis, through a mutation of the gene for gulonolactone-oxidase (*Gulo*) in the ancestor of man [11]. This fact led to the hypothesis that these two evolutionary events may have been connected [12]. The loss of endogenous ascorbate synthesis rendered our ancestors susceptible to scurvy, a condition characterized by the impairment of collagen synthesis and ensuing loss of connective tissue integrity with hemorrhagic blood loss being the most frequent cause of death.

During ECM impairment, caused by extended periods of micronutrient deficiency, the presence of the apo(a) macromolecule with a primary structure larger than collagen, with antifibrinolytic properties and with high binding affinity to ECM components could have functioned as a repair molecule compensating for the structural ECM impairment caused by hypoascorbemia and scurvy.

## Vitamin C affects Lp(a) in transgenic mice

To test this hypothesis, we developed a transgenic mouse model that mimics human metabolism with two distinct genetic features: the lack of endogenous ascorbate synthesis and the expression of human Lp(a). Specifically, we investigated whether the loss of vascular integrity due to prolonged hypoascorbemia, results in the deposition of Lp(a) molecules inside the vascular wall and consequent atherosclerosis. Moreover, we investigated whether dietary supplementation with high levels of ascorbate can prevent this pathological process.

### Material and methods

#### Mouse colony

All mice were maintained in microisolator cages under pathogen-free conditions on a 12-hour light/ dark cycle. All procedures were conducted according to humane and customary care and use of experimental animals, and followed a protocol approved by internal institutional animal safety review committee. *Gulo*<sup>-/-</sup> mice that are unable to synthesize their own ascorbate (vitamin C) had this nutrient included in a diet. Unless indicated, vitamin C was provided in double distilled drinking water containing 150 mg/L ascorbic acid (Sigma) and 0.01 mM EDTA (Sigma) in addition to 10 g/L of sucrose. Water was changed twice a week. In addition, vitamin C was provided in food fortified with 500 ppm L-ascorbyl-polyphosphate, milled at Test Diet® as a Modified Custom Lab Diet #5A38. During the study mice consumed on average 4 g of food and drank about 5 mL of water daily.

#### Generating human Lp(a)<sup>+</sup>; *Gulo*<sup>-/-</sup> mice

*The founder mice strains used for cross breeding BALB/cBy-Gulo*<sup>-/-</sup> mice: The strain, BALB/cBy-*Gulo*<sup>sfx</sup>/J was a spontaneous mutation, mapped to the gulonolactone oxidase locus, a gene necessary for vitamin C synthesis. The *Gulo*<sup>-/-</sup> strain mouse was generated from heterozygous *Gulo*<sup>+/-</sup> breeders obtained from The Jackson Laboratory (Sacramento, CA). The Human *apo(a)* mouse was obtained from the Mutant Mouse Regional Resource Centers (MMRRC) (Columbia, MO) supported by NIH. The Human *apo B-100* mouse was obtained from Taconic Farms, Inc. (Hudson, NY) under an academic research agreement.

*Cross breeding for Gulo*<sup>-/-</sup>; Lp(a)<sup>+</sup>mice: Human *apo(a)* and Human *apoB-100* mice wild type for

the *Gulo* locus were bred to *Gulo*<sup>-/-</sup> mice separately to generate two experimental founder mice strains: *Gulo*<sup>-/-</sup>; human *apo(a)*<sup>+</sup> and *Gulo*<sup>-/-</sup>; human *apoB-100*<sup>+</sup>. Subsequently, the newly generated mice breeders of both strains were crossed to generate the new mice strain: *Gulo*<sup>-/-</sup>; human *apo(a)*<sup>+</sup>; human *apoB-100*<sup>+</sup> named as "*Gulo*<sup>-/-</sup>; Lp(a)<sup>+</sup>" strain.

#### Genotyping

Genotyping for the *Gulo* locus and its homozygosity as well as for the presence of human *apoB-100* and human *apo(a)* was performed via Taqman FAM Probe Real Time-PCR at Transnetyx (Cordova, TN) upon tail clip tissue derived DNA obtained using standard DNA isolation and PCR techniques.

#### Transgene expression at the level of protein

Presence of apoB-100 protein in mice serum was determined by using Assaypro (St. Charles, MO) AssayMax Human Apolipoprotein enzyme immunoassay, which is human apoB-100 specific and does not cross-react with mouse apoB, or with any other of the apolipoproteins (Apo AI, Apo C, Apo E). Apo(a) protein in mice serum was determined by using the IBL International GmbH (Hamburg, Germany) Lp(a) Enzyme immunoassay, which is human apo(a) specific and does not cross-react with plasminogen or LDL. All known isoforms of apo(a) can be detected. Serum apo(a) protein was present in *apo(a)* gene containing *Gulo*<sup>-/-</sup> mice, *apo(a)* and human *apoB-100* gene containing *Gulo*<sup>-/-</sup> mice, but not *Gulo*<sup>-/-</sup> mice without the transgene nor in *Gulo*<sup>-/-</sup> mice with only Human apoB-100. These results confirm expression and translation of the human transgene *apo(a)* to serum protein apo(a).

The apo(a) protein was present in serum of both male and female mice before puberty. Male mice after puberty have significantly or completely repressed apo(a) protein expression, possibly due to elevated testosterone levels. It has been previously shown that apo(a) expression in male mice may be restored via castration, continuous infusion of growth hormone via osmotic pump, or by biochemical modulation by dietary, chemical, or biological inducers [13].

The Lp(a) particles are composed of human apo(a) protein linked to human apoB-100 (LDL)

## Vitamin C affects Lp(a) in transgenic mice

by disulfide bonds. The presence of Lp(a) particle in the *Gulo*<sup>-/-</sup>; Lp(a)<sup>+</sup> transgenic mice serum was confirmed using the electrophoresis method by Helena (Beaumont, TX) SPIFE Cholesterol Profiling [14]. According to this method, the Lp(a)-cholesterol band is located at a specific migration distance between LDL-cholesterol and HDL-cholesterol bands. It is found in *Lp(a)* gene(s) containing *Gulo*<sup>-/-</sup> mouse sera, but not in the sera of *Gulo*<sup>-/-</sup>; *apo(a)*<sup>+</sup>; *Gulo*<sup>-/-</sup>; or *apoB-100*<sup>+</sup>; *Gulo*<sup>-/-</sup>; mice confirming that the presence of both human *apoB-100* and *apo(a)* are necessary for disulfide linked serum Lp(a). Human *apo(a)* alone is insufficient to produce Lp(a) and it does not link to mouse LDL. In addition, we conducted Immunofixation Electrophoresis (IFE) using human specific anti-*apo(a)* and anti-*apoB-100* antibodies at Health Diagnostic Laboratory, Inc. (Richmond, VA). Additional particle data in conjunction with the lipoprotein cholesterol load data provided comprehensive confirmation of the presence of *apo(a)* protein, human *apoB-100* protein, and disulfide linked Lp(a) in these mice sera.

### *Experimental ascorbate regimens*

Mice of nearly equal age were kept on 30 mg/L or 60 mg/L vitamin C, 0.01 mM EDTA in drinking water, with 10 g sucrose/L and no additional vitamin C in food to provide approximately 0.15 mg/day vitamin C or 0.30 mg/day vitamin C respectively for 12 weeks. Mice of nearly equal age were kept on regular distilled water and unsupplemented food to cause full deprivation of vitamin C for 6 weeks. Mice unable to generate vitamin C die within 8 weeks of full deprivation. Full supplementation of 2.75 mg vitamin C per day was administered as described above. All mice were on average 40 weeks old at end-point.

### *Ascorbate determinations*

Serum levels of vitamin C were obtained using the Biovision (Milpitas, CA) Ferric Reducing Ascorbate Assay (FRASC) Kit. Serum was obtained from plasma drawn through cardiac puncture before arterial harvest.

### *Cholesterol determinations*

Individual serum total cholesterol (mg/dL) was obtained using the Biovision (Milpitas, CA) Cholesterol/Cholesteryl Ester Quantitation Colorimetric Kit II. Quantitative values for lipoprotein fraction cholesterol (mg/dL) were deter-

mined by applying relative fraction data from the lipoprotein cholesterol profiles to total cholesterol values.

### *Triglyceride determination*

Serum triglycerides were obtained using the Biovision (Milpitas, CA) Triglyceride Quantitation Colorimetric/Fluorimetric Kit. Triglyceride cargo was considered a different value than VLDL cholesterol cargo or VLDL mass or particle number.

### *Mouse ApoB determination*

Serum mouse ApoB concentrations were determined via Cloud-Clone Corp (Houston, TX) ELISA Kit for Apolipoprotein B (APOB) specific to mouse (*Mus musculus*) applying a correction for dilution factor.

### *Histology*

Mice arteries were harvested and trimmed in ice cold PBS under a dissection microscope after plasma draw. Brachiocephalic, left carotid, left subclavian, right carotid, and right subclavian were all taken on the arch with thoracic aorta in one piece and fixed in 10% neutral buffered formalin and embedded in paraffin. En face sections to the entire tree were attempted to keep all pieces in plane. Hematoxylin and Eosin and Elastic Van Gieson stains were performed at IDEXX (Sacramento, CA). Special attention was paid to those specimens that exhibited endothelial denudation, intimal hyperplasia, subendothelial thickenings, elastin disorganization, atherosclerotic plaques, and pre-aneurysmal structures. These blocks were then sectioned and stained for human *apo(a)*, human Apo B-100, and fibrinogen by typical immunohistochemistry with positive and negative antibody controls at Histotox Labs, Inc. (Boulder, CO).

### *Statistical analysis*

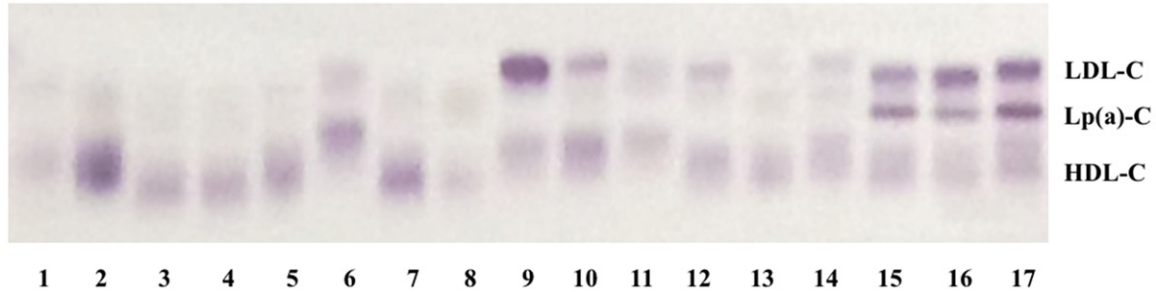
The results were expressed as means  $\pm$  SD, as indicated in the results. Data was analyzed by independent sample "t" test.  $p < 0.05$  were considered as statistically significant.

## **Results**

### *A transgenic mouse model to study human atherosclerosis*

We developed a new transgenic mouse strain expressing human Lp(a) and lacking L-gulono-

## Vitamin C affects Lp(a) in transgenic mice



**Figure 1.** Lipoprotein cholesterol profiling in transgenic mice of different genetic backgrounds. Serum from different randomly chosen previously genotyped *Gulo*<sup>-/-</sup> mutant male and female background strain mice, single human transgenic mice (*apo(a)* and *apoB-100*), and double human transgenic mice (*Gulo*<sup>-/-</sup>; *apo(a)*+; *apoB-100*+, for *Gulo*<sup>-/-</sup>; Lp(a)+). Mice serum was resolved for lipoproteins via electrophoresis and their cholesterol cargo visualized as described in 'Material and Methods'. Expression of apo(a) or h-apoB-100 protein was determined by ELISA previously as described in 'Material and Methods.' the alpha band close to the bottom is HDL-cholesterol. The beta band closest to the top is LDL-cholesterol. The strong pre-beta band seen below LDL in lanes 15-17 is Lp(a)-cholesterol. Lanes: 1 and 2) male *Gulo*<sup>-/-</sup>. 3 and 4) female *Gulo*<sup>-/-</sup>. 5 and 6) male *Gulo*<sup>-/-</sup>; *apo(a)*+. 7 and 8) female *Gulo*<sup>-/-</sup>; *apo(a)*+. 9 and 10) male *Gulo*<sup>-/-</sup>; *apoB-100*+. 11 and 12) female *Gulo*<sup>-/-</sup>; *apoB-100*+. 13 and 14) male *Gulo*<sup>-/-</sup>; *apo(a)*+; *apoB-100*+. 15, 16, and 17) female *Gulo*<sup>-/-</sup>; *apo(a)*+; *apoB-100*+.

**Table 1.** Serum levels of ascorbate, lipids and lipoproteins in *Gulo*<sup>-/-</sup> and *Gulo*<sup>-/-</sup>; Lp(a)+ mice under different dietary ascorbate supplementation

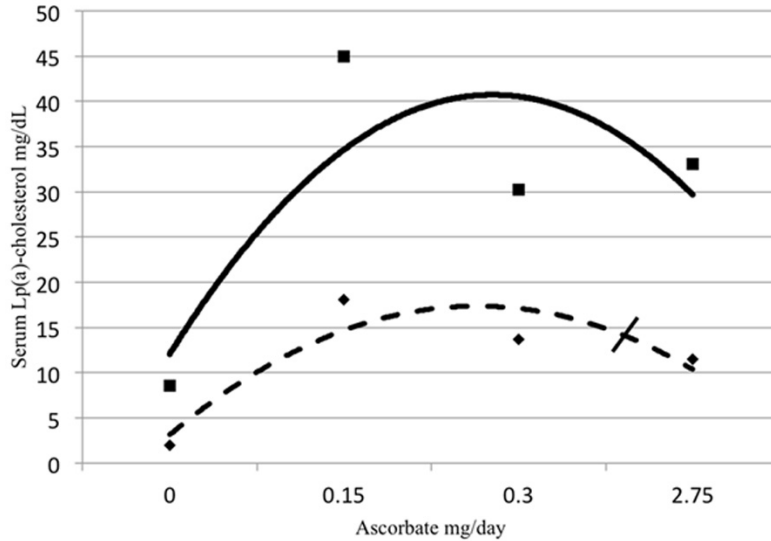
Mice Type		<i>Gulo</i> <sup>(-/-)</sup>		<i>Gulo</i> <sup>(-/-)</sup> ; Lp(a)+		
Dietary Ascorbate		2.75 mg/day	0 mg/day*	0.15 mg/day	0.30 mg/day	2.75 mg/day
Serum Ascorbate	M	ND	<b>3.0±6.9</b>	<b>5.1±3.6</b>	<b>10.4±3.1</b>	50.0±12.5
	F	ND	<b>7.9±8.5</b>	<b>3.2±1.9</b>	<b>5.0±1.7</b>	53.5±10.1
Total-C	M	101.6±6.4	<b>140.6±17.0</b>	112.3±25.9	124.3±26.4	112.6±7.9
	F	88.2±11.3	139.3±25.4	103.2±16.6	96.3±23.6	105.1±23.3
LDL-C	M	7.5±0.71	<b>97.4±14.3</b>	24.6±15.9	28.9±11.2	26.6±13.7
	F	7.3±0.5	<b>84.5±26.9</b>	14.8±6.3	19.2±10.3	20.3±9.1
HDL-C	M	60.3±5.1	<b>41.2±7.9</b>	62.6±28.6	67.9±18.1	68.0±13.2
	F	47.1±5.7	46.2±11.7	38.3±9.8	39.8±10.1	45.2±10.5
Lp(a)-C	M	0	<b>2.0±1.7</b>	<b>18.1±10.5</b>	13.7±3.9	11.5±4.4
	F	0	<b>8.6±5.7</b>	<b>45.0±12.6</b>	30.2±13.3	33.1±10.9
TG	M	231.2±58.0	<b>320.3±71.8</b>	<b>562.7±134.4</b>	<b>590.4±94.7</b>	445.5±104.3
	F	199.4±82.3	411.0±136.4	436.7±107.7	354.0±77.9	423±125.0
h-apo(a)	M	0	<b>0</b>	<b>26.1±43.5</b>	<b>12.3±13.2</b>	0.3±0.91
	F	0	<b>6.7±8.9</b>	<b>100.4±36.9</b>	69.3±28.5	75.5±21.6
h-apoB	M	0	79.1±22.9	97.9±83.1	<b>93.2±42.5</b>	60.1±31.7
	F	0	106.4±50.8	<b>70.8±28.1</b>	52.5±25.9	41.5±16.5

Total-C = Total-cholesterol; LDL-C = low-density lipoprotein-cholesterol; HDL-C = high-density lipoprotein-cholesterol; Lp(a)-C = lipoprotein(a)-cholesterol; TG = triglycerides; h-apo(a) = human-apolipoprotein(a); h-apoB = human-apolipoprotein B; ND = not determined. Lipid and lipoproteins expressed in mg/dL. Ascorbate expressed in μmol. \*Average for n = 7-9 mice, all other groups average of n = 10-12. \*Results after 6 weeks on a diet. Values in bold represent statistically significant (p<0.05) differences when compared to the values in mice supplemented with high levels of ascorbate.

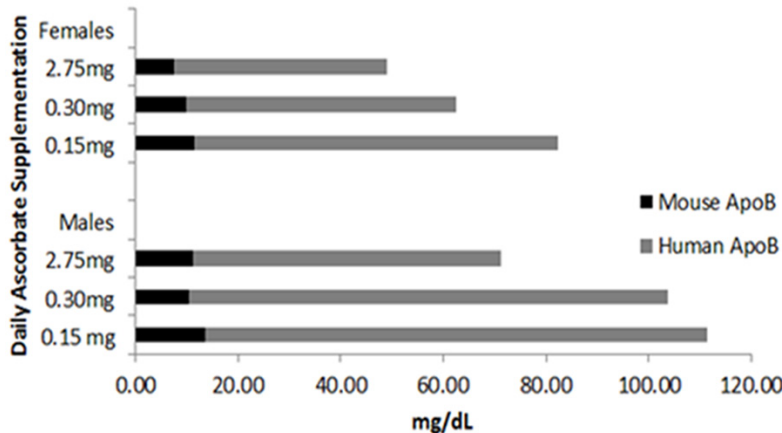
lactone oxidase (*Gulo*<sup>-/-</sup>) by stepwise cross breeding as described in 'Material and Methods'. Both human proteins, apo(a) and ApoB-100, were detected in the sera of double transgenic *apo(a)*+; *apoB-100*+; *Gulo*<sup>-/-</sup> mice. The presence of assembled Lp(a) particles was confirmed in the serum of this mouse strain

with electrophoretic lipoprotein separation and cholesterol visualization (**Figure 1**), as described in 'Material and Methods'. Transgenic mice models expressing human Lp(a) had been developed before [15, 16], but these animal models differed from human metabolism with respect to endogenous ascorbate synthesis.

## Vitamin C affects Lp(a) in transgenic mice



**Figure 2.** Response of serum Lp(a) levels in *Gulo*<sup>-/-</sup>; Lp(a)<sup>+</sup> mice to different dietary ascorbate supplementation. Serum ascorbate and Lp(a)-cholesterol levels in male and female *Gulo*<sup>-/-</sup>; Lp(a)<sup>+</sup> mice were measured as described in 'Material and Methods'. The trends for male and female values were evaluated using the averaged Lp(a) determinations from the 6 week washout experiment for the zero ascorbate dose and 12 weeks hypoascorbemia experiment for the other ascorbate doses. A parabolic or biphasic curve for Lp(a) concentration appears in males (broken line) and female mice (solid line) across the ascending dose range of ascorbate.



**Figure 3.** Mouse apoB and human apoB-100 serum levels in male and female transgenic *Gulo*<sup>-/-</sup>; Lp(a)<sup>+</sup> mice. Native mouse apoB and transgenic human apoB-100 protein were measured separately in the serum of a new transgenic strain of *Gulo*<sup>-/-</sup>; Lp(a)<sup>+</sup> mouse. In the serum of these mice, the majority of circulating apoB was human apoB-100, and a significant amount was detected in the Lp(a) fraction. In this animal model, human apoB-100 serum levels varied with dietary ascorbate intake in a manner similar to Lp(a).

### Ascorbate modulates lipid and lipoprotein plasma levels

We selected four levels of dietary ascorbate supplementation, which correspond to 0 mg,

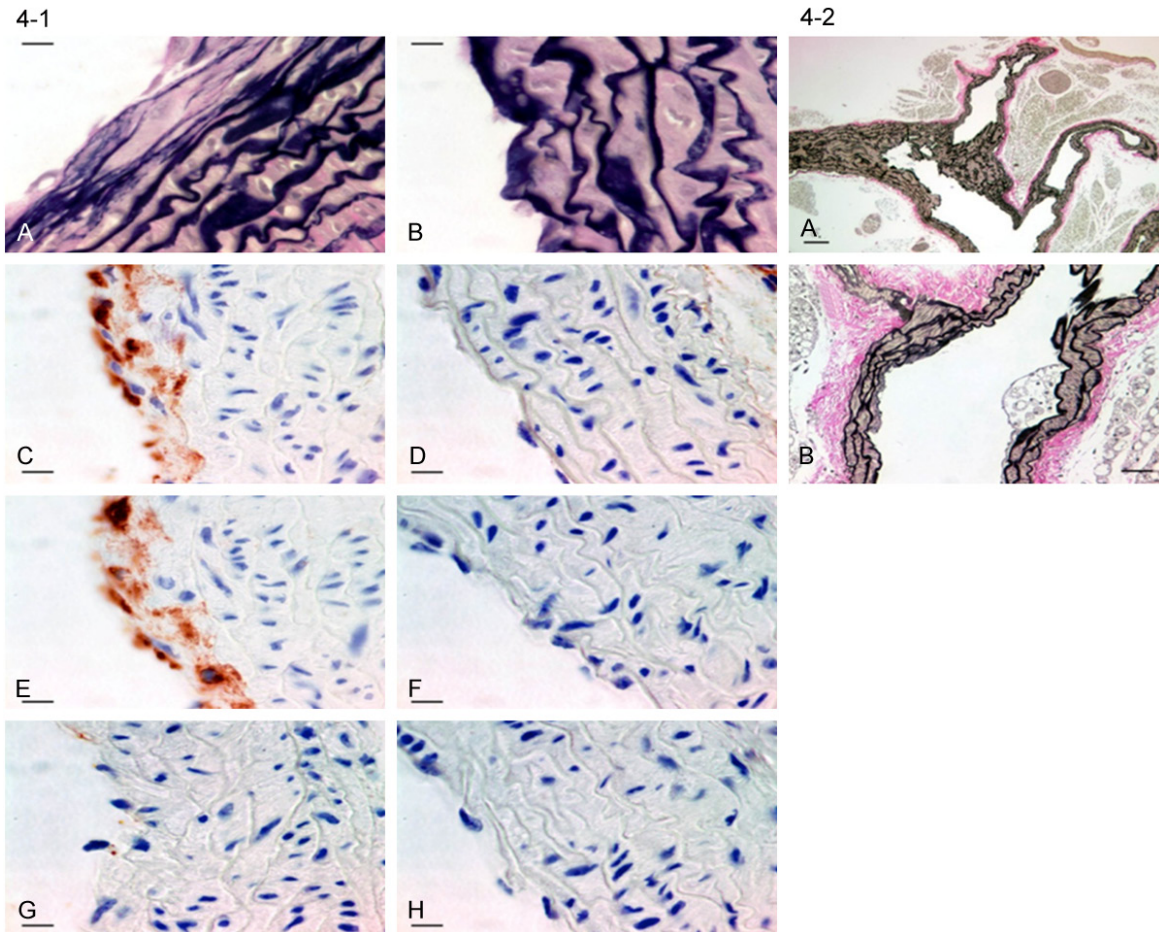
0.15 mg, 0.3 mg and 2.75 mg of ascorbate intake per mouse per day delivered in food and water (see 'Material and Methods'). These ranges of ascorbate supplementation were chosen to modulate different states of connective tissue integrity, particularly in the vasculature.

As **Table 1** indicates, dietary intake of ascorbate correlated with the serum levels of this vitamin, with no significant gender differences. The *Gulo*<sup>-/-</sup>; Lp(a)<sup>+</sup> mice on the highest ascorbate supplementation (2.75 mg/day) had average ascorbate serum levels of above 50  $\mu$ M, which is close to the serum values reported in wild type mice (about 63  $\mu$ M) with no gender differences. After six weeks of complete dietary ascorbate deprivation, the ascorbate serum levels in male mice decreased to 3  $\mu$ M. However, in females on a zero ascorbate diet we observed some variation of ascorbate serum levels with 3 animals at 0  $\mu$ M and a group average of 7.9  $\mu$ M.

Normal, wild-type mice with endogenous production of ascorbate have a lipoprotein profile inverse to the human metabolism with high-density lipoproteins (HDL) being the primary cholesterol carrier in plasma and low-density lipoprotein (LDL) and very-low-density-lipoprotein (VLDL) playing a secondary role. In contrast, *Gulo*<sup>-/-</sup>; Lp(a)<sup>+</sup> mice on a diet completely depleted of ascorbate

had significantly elevated serum total cholesterol and LDL becomes the main lipoprotein fraction. High dietary ascorbate supplementation results in a significant decrease of total cholesterol and LDL-cholesterol.

## Vitamin C affects Lp(a) in transgenic mice



**Figure 4.** Ascorbate deprivation promotes the deposition of Lp(a) in the vascular wall. Figure 4-1. Immunostains of the aortic root sections from female *Gulo*<sup>-/-</sup>; Lp(a)<sup>+</sup> mice after 6 weeks of ascorbate deprivation (A, C, E, G) and under supplementation with 2.75 mg of ascorbate daily (B, D, F, H). A and B - Elastic Van Gieson staining, C and D immunostaining for human apo(a), E and F immunostaining for human apoB-100 and G and H for fibrinogen, Scale bars = 10 μm. Figure 4-2. Elastic Van Gieson stain of an atherosclerotic plaque formed in the cervical branches of the left subclavian artery shown in low and high magnification (Figure 4-2A and B). The picture was taken from the artery of a 10 month old female *Gulo*<sup>-/-</sup>; Lp(a)<sup>+</sup> mouse after 12 weeks on a low ascorbate diet (0.15 mg/dL). Figure 4-2A: Scale Bar = 200 μm. 4-2 B: Scale Bar = 50 μm.

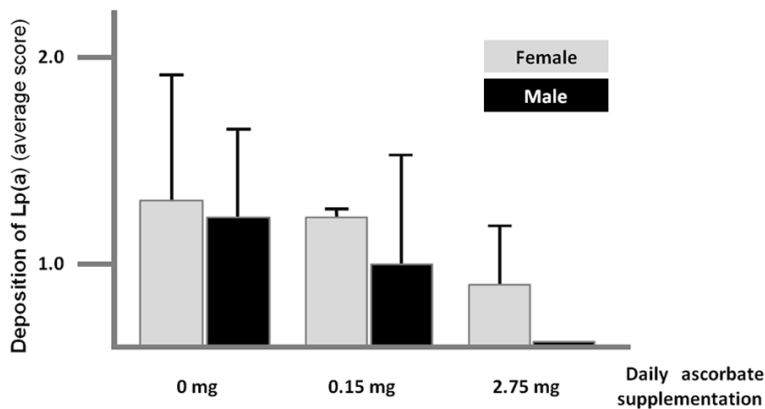
The transgenic *Gulo*<sup>-/-</sup>; Lp(a)<sup>+</sup> animal model allowed us for the first time to study the impact of dietary ascorbate intake in an animal unable to endogenously synthesize ascorbate and to measure its Lp(a) serum levels. Our data showed a significant increase in serum Lp(a) levels with a decrease of dietary ascorbate intake. However, with zero ascorbate intake over 6 weeks, a condition mimicking human scurvy, serum Lp(a) levels dropped significantly, probably due to a decrease of protein synthesis in this metabolic condition. We also observed significant gender differences in serum Lp(a) levels with female mice showing significantly higher levels than males of corresponding ascorbate intake and age (**Figure 2**).

Since apoB containing LDL is also a constituent of mouse metabolism, we differentiated the serum levels of genuine mouse apoB and human apoB-100 in transgenic *Gulo*<sup>-/-</sup>; Lp(a)<sup>+</sup> mice. The great majority of apoB circulating in serum of these transgenic mice is human apoB-100, with a significant portion detected in the Lp(a) fraction. As with Lp(a) serum levels, human apoB-100 serum levels in this animal model were also dependent on dietary ascorbate intake (**Figure 3**).

### *Ascorbate deficiency triggers deposition of Lp(a) in the vascular wall*

Ascorbic acid is essential for the hydroxylation of lysine and proline, a precondition for opti-

## Vitamin C affects Lp(a) in transgenic mice



**Figure 5.** Extent of Lp(a) deposition in vascular wall is inversely related to dietary ascorbate. The quantitative evaluation of histological immunostains for apo(a) and apoB-100 in the vascular wall is expressed as a score value calculated by multiplying the immunostained area by its intensity. The proximal, ascending, descending and thoracic part of the aorta was examined, including its branching vessels. Only areas in which apo(a) and apoB-100 were co-localized and formed clear deposits were included. A “0” score represents no deposits, “1” minimal deposit(s), “2” moderate deposit(s), and a score of “3” represents extensive deposit(s).

num crosslinking of collagen and elastin and, thus, for maintaining the integrity of the vascular wall [17]. The *Gulo*<sup>-/-</sup>; Lp(a)<sup>+</sup> model enables the studying of the connection between ascorbate deficiency and atherogenesis, with one of its hallmarks being Lp(a) deposition in the vascular wall. As in human scurvy, complete dietary ascorbate deprivation in this mouse model leads to marked alteration in the structural integrity of vascular connective tissue. Disruption of the endothelial layer, impairment of the basement membrane and the structural disintegration of the ECM paralleled the development of scurvy [18].

We found that the development of early atherosclerosis was accompanied by the deposition of Lp(a) in the intima and deeper layers of the vascular wall. Age-matched mice with high amounts of dietary ascorbate supplementation did not develop atherosclerosis with no Lp(a) detectable in the vascular wall. **Figure 4-1** shows microscopic pictures of sequential histological slices from the aortic root of ascorbate deprived (**Figure 4-1A, 4-1C, 4-1E, 4-1G**) and fully supplemented animals (**Figure 4-1B, 4-1D, 4-1F, 4-1H**). In the artery wall of ascorbate deficient animals, elastin degeneration (**Figure 4-1A**) concurs with the deposition of Lp(a) molecules, as identified by immunohistochemical co-localization of apo(a) (**Figure 4-1C**) and

apoB-100 (**Figure 4-1E**) and fibrinogen (**Figure 4-1G**).

The affinity of apo(a) to different ECM molecules had previously been established *in vitro*, however, the *in vivo* conditions under which such events take place had not been elucidated thus far. Here we show that ascorbate deficiency is a metabolic condition under which Lp(a) accumulates in structurally impaired areas of the vascular wall. Due to its homology with plasminogen, apo(a) has been shown to have strong binding affinity to fibrinogen and fibrin which is corroborated by the co-localization of apo(a) and fibrinogen (**Figure 4-1G**).

Our results also show that after 12 weeks of hypoascorbemia, large atherosclerotic lesions can develop. Just as with human atherosclerosis, these lesions develop preferentially at sites of elevated hemodynamic pressure and high shear stress, i.e. in the proximal aorta and the adjacent branching regions (**Figure 4-2A, 4-2B**).

### Quantification of Lp(a) deposition in the vascular wall with different levels of dietary ascorbate

Studies in human CVD have shown that the deposition of Lp(a) inside the vascular wall parallels the extent of atherosclerotic deposits [4]. However, in these studies no ascorbate levels were determined. Here we show that both the stage of atherosclerotic lesions, as well as the deposition of Lp(a) inside the vascular wall of *Gulo*<sup>-/-</sup>; Lp(a)<sup>+</sup> mice, are inversely correlated with dietary intake of ascorbate (**Figure 5**). The Lp(a) deposition was assessed by a grading system using an ascending deposition scale of 0 to a maximum score of 3. Suboptimal dietary intake of ascorbate (0.15 mg/dL) or the complete dietary absence of this vitamin (0 mg/dL) led to a significant accumulation of Lp(a) inside the vascular wall in both genders.

In contrast, the highest level of ascorbate supplementation used in this study (2.75 mg/dL),

## Vitamin C affects Lp(a) in transgenic mice

resulted in a significant reduction of Lp(a) deposition in the vascular wall of these mice. In female *Gulo*<sup>-/-</sup>; Lp(a)+ mice, minor deposition of Lp(a) inside the vascular wall was observed (score <1). In males on high dietary ascorbate, deposition of Lp(a) inside the vascular wall was completely prevented (score = 0).

### Discussion

While naturally occurring atherosclerosis is the exception in lower mammals, it is the primary cause of death in *Homo sapiens*. Two genetic features of human metabolism that have been implicated in the development of CVD are, firstly, ascorbate deficiency due to the loss of endogenous ascorbate synthesis and, secondly, the appearance of Lp(a). Since both these genetic events occurred around the same time, about 40 million years ago, it has been suggested that these events may be connected [12]. Lipoprotein(a), as a formidable repair molecule, would counteract the potentially fatal consequences of ascorbate deficiency on the extracellular matrix.

Due to the lack of a suitable animal model, the connection between ascorbate deficiency and Lp(a) could, thus far, not be studied *in vivo*. Previous animal models could address these two metabolic features only in a separate way. *Gulo*<sup>-/-</sup> mice on ascorbate deficient diet displayed structural changes of the vascular wall accompanied by elevated cholesterol levels [19], but these animals did not express Lp(a). Transgenic mice expressing human Lp(a) required a high-fat diet to induce atherogenesis, most likely because the endogenous synthesis of ascorbate prevented the impairment of the vascular wall as a precondition for atherogenesis [15]. By establishing the *Gulo*<sup>-/-</sup>; Lp(a)+ mouse model the interaction of these two metabolic features in atherogenesis could be studied for the first time. We established that in *Gulo*<sup>-/-</sup>; Lp(a)+ mice, dietary ascorbate deficiency is associated with increases of total cholesterol, LDL-cholesterol and Lp(a). Suboptimal dietary amounts of ascorbate lead to a significant increase of serum Lp(a) levels, whereas complete dietary depletion of ascorbate is associated with decreased serum levels of this lipoprotein.

Thus, during stages of hypoascorbemia, Lp(a) becomes a significant contributor to the overall

lipoprotein profile in this animal model, whereas with Lp(a) decline in complete ascorbate depletion (scurvy), LDL rises to become the primary lipoprotein fraction.

We also observed a significant gender difference with respect to the serum levels of Lp(a). Female mice had consistently much higher Lp(a) levels compared to their male counterparts at corresponding age and dietary ascorbate. The *Gulo*<sup>-/-</sup>; Lp(a)+ model now also enables the studying of the underlying cause of this gender difference in detail, potentially revealing novel Lp(a) phenomena.

These findings from an animal model replicating human metabolism with respect to two key metabolic factors may provide new insights in the relation of serum risk factors and atherogenesis in man. Our results suggest that elevated serum levels of Lp(a) may not be considered a primary cause of CVD but rather a consequence of the disintegration of vascular connective tissue as part of the already developing disease.

Of particular interest was the question whether Lp(a) molecules are deposited in the vascular wall during dietary ascorbate deficiency. The morphological changes of the vascular wall during hypoascorbemia and scurvy are characterized by the breakdown of the ECM and a loss of the endothelial barrier function. Large endothelial gaps allow the influx of macromolecules into the vasculature that are normally excluded by an intact endothelium.

Our data show that the accumulation of Lp(a) occurs in the vascular wall of mice during hypoascorbemia and scurvy, but is essentially absent in animals on high dietary ascorbate. Of particular significance is the fact that these deposits developed without any challenges by a high-lipid diet, which is required to induce atherosclerosis in other animal models. These data indicate that the structural impairment of the vascular wall in hypoascorbemia and scurvy by itself is sufficient for the deposition of Lp(a) and the development of atherosclerosis.

It is noteworthy that Lp(a) accumulated in the vascular wall of ascorbate depleted (scurvitic) animals, despite ascorbate serum levels being substantially decreased in this extreme metabolic condition. This suggests a preferential uptake and accumulation of Lp(a) at sites of



## Vitamin C affects Lp(a) in transgenic mice

highest metabolic needs, exemplified by the extreme challenge to vascular integrity during scurvy.

Our data imply that Lp(a) functions as a mobile repair molecule accumulating at sites of vascular lesions during hypoascorbemia. These observations can also explain the avid binding of the apo(a) molecule to ECM components to counteract structural matrix impairment during hypoascorbemia. It is noteworthy that Lp(a) accumulated in the vascular wall of hypoascorbemic and scorbutic animals almost primarily extracellularly, suggesting a role of intact Lp(a) molecules inside the structurally impaired vascular wall. These results corroborate with similar findings in human atherosclerosis [4, 20].

Moreover, the unique structure of the apo(a) molecule with its homology to plasminogen offers an advantage by counteracting fibrinolysis and hemorrhagic blood loss in scurvy. By occupying plasminogen binding sites, apo(a) prevents plasmin degradation of fibrin [7]. The co-localization of Lp(a) and fibrinogen inside the vascular lesions we observed, concurs with this potential role. In addition, by associating with LDL, the Lp(a) molecule also provides essential lipid substrates for cellular repair.

This new *Gulo*<sup>-/-</sup>; Lp(a)<sup>+</sup> mouse model provides an opportunity to study a new concept of human atherogenesis based on metabolic conditions unique to our species. The prevailing current model of atherogenesis postulates hyperlipidemia [21] and other systemic risk factors [22, 23] as the precondition for the initiation of atherosclerosis. These models, however, would logically lead to generalized atherosclerosis and peripheral vascular disease.

This mouse model can now expand our understanding of human CVD compatible with its prevailing manifestation at sites of increased hemodynamic stress. Moreover, it can provide new insight into the occurrence of CVD in non-lipidemic patients.

Our study design mimics important events during the evolution of man. After the loss of endogenous ascorbate synthesis, the survival of our ancestors was repeatedly threatened through epidemics of scurvy. Individuals expressing Lp(a) may have had an evolutionary advantage by effectively counteracting hemorrhagic blood loss through a scurvy-impaired vascular wall.

The development of this new mouse model and the results of this study should encourage further research on the role of dietary factors, like ascorbate, as a contributing factor to vascular health.

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### Disclosure of conflict of interest

None.

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