TITLE PAGE

Title. APOA-1Milano muteins, orally delivered via genetically modified rice, show anti-atherogenic and anti-inflammatory properties *in vitro* and in *Apoe*$^{-/-}$ atherosclerotic mice

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Keywords. Atherosclerosis; inflammation; nutraceutic; apolipoprotein A-1

Structured Abstract

Background. Atherosclerosis is a slowly progressing, chronic multifactorial disease characterized by the accumulation of lipids, inflammatory cells, and fibrous tissue that drives to the formation of asymmetric focal thickenings in the *tunica intima* of large and mid-sized arteries. Despite the high therapeutic potential of ApoA-1 proteins, the purification and delivery into the disordered organisms of these drugs is still limited by low efficiency in these processes.

Methods and Results. We report here a novel production and delivery system of anti-atherogenic APOA-1Milano muteins (APOA-1M) by means of genetically modified rice plants. APOA-1M, delivered as protein extracts from transgenic rice seeds, significantly reduced macrophage activation and foam cell formation *in vitro* in oxLDL-loaded THP-1 model. The APOA-1M delivery method and therapeutic efficacy was tested in healthy mice and in *Apoe-/-* mice fed with high cholesterol diet (Western Diet, WD). APOA-1M rice milk significantly reduced atherosclerotic plaque size and lipids composition in aortic sinus and aortic arch of WD-fed *Apoe-/-* mice as compared to wild type rice milk-treated, WD-fed *Apoe-/-* mice. APOA-1M rice milk also significantly reduced macrophage number in liver of WD-fed *Apoe-/-* mice as compared to WT rice milk treated mice.

Translational impact. The delivery of therapeutic APOA-1M full length proteins via oral administration of rice seeds protein extracts (the 'rice milk) to the disordered organism, without any need of purification, might overcome the main APOA1-based therapies' limitations and improve the use of this molecules as therapeutic agents for cardiovascular patients.

Abbreviations

CVD, cardiovascular diseases; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoproteins cholesterol; gDNA, genomic DNA; WT, wild type; oxLDL, oxidized LDL; MCP-1, monocyte chemoattractant protein-1; WD, Western diet.

MANUSCRIPT TEXT

1. Introduction

The most common underlying cause of cardiovascular diseases (CVD) is atherosclerosis, a slowly progressing, chronic inflammatory disease in which lesions called plaques are formed in focal areas of large and mid-size arteries[1]. The atherogenic process starts as a complex result of activation of endothelial cells, that, once exposed to injurious stimuli (such as dyslipidemias and pro-inflammatory mediators), change their permeability[2] triggering subendothelial retention of cholesterol-containing plasma lipoproteins[3] and recruiting innate immunity cells that ultimately lead to intraplaque inflammation and towards a pro-thrombotic state[4]. Increased blood total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) as well as the so called atherogenic lipid triad (increased very low density lipoprotein, VDLD; increased small dense low density lipoproteins; reduced high density lipoproteins cholesterol, HDL-C) appear to be relevant for cardiovascular diseases[5]. The early identification and management of modifiable risk factors, primarily those contributing to dyslipidaemias, is the first prevention line for cardiovascular diseases[6]. Since plasma levels of blood HDL-C inversely correlated with risk of coronary heart disease[7], the anti-inflammatory and atheroprotective effects of HDL-C have been deeply investigated[8]. Among the protein components of HDLs, there have been interest in the therapeutic potential of the ApoA-1, which is able to stimulate reverse cholesterol transport, thus facilitating the removal of free cholesterol from peripheral tissues, especially from arterial walls resulting in an anti-atherogenic effect[9,10]. Moreover, the importance of ApoA-1 in atherosclerotic process has also been demonstrated by the observation that anti-ApoA-1 antibodies increased plaque vulnerability in *Apoe*$^{-/-}$ mice via a TLR2 and TLR4 pathways[11,12] and serum levels of anti-ApoA-1 auto-antibodies correlate with more vulnerable plaques in humans[11]. Although several studies on animal models of atherosclerosis demonstrated the potential of using HDLs as therapeutic strategy, clinical trials have substantially failed to show significant reduction in atheroma volume when recombinant or mimetic HDLs were administered by infusion[13]. APOA-1Milano is a naturally occurring mutation of human ApoA-1

which results in reduced HDL-cholesterol levels, also in APOA-1_{Milano} transgenic mice[14], with a concomitant low prevalence of CVD[15]. The APOA-1Milano protein has been demonstrated to be effective in rapidly reducing atherosclerotic plaques in mice, rabbit and porcine models [10,16-18] and in clinical trials[19]. Nevertheless, the therapeutic use of these lipoproteins has not been fully exploited yet probably because of the very low efficiency of production and/or purification of these lipoproteins.

We report here the effects, evaluated in appropriate experimental *in vitro* and *in vivo* models of dyslipidemia and atherosclerosis, of a novel drug delivery system, without any need of purification, of anti-atherogenic ApoA-1Milano molecules, by means of their synthesis in seeds of genetically modified rice plants administered to the disordered organism by oral gavage in form of seed extract, the "APO milk".

2. Materials and Methods

Standard methods are detailed in the online Supplementary Methods.

2.1 Genetically modified rice plants and rice protein extract.

Genetically modified rice milk (APOA-1M) was produced as indicated in patent n° PCT/IB2006/054948. The rice milk was provided as lyophilized powder by GRG Gene Technology SA (Minusio, Switzerland). Non-genetically modified rice milk of the same variety (Rosa-Marchetti) was used as a control. For *in vitro* experiments rice milk was handled in sterile conditions, resuspended at a concentration of 2,5 g/mL in Phosphate Buffer Saline (PBS) and additioned with Zell-Shield (Minerva Biolabs). For *in vivo* experiments, rice milk was resuspended at a concentration of 2,5 g/mL in sterile water.

2.2 Genetic modification and molecular analysis of transgenic rice plants.

The engineered plasmids were introduced in *Agrobacterium tumefaciens* strain EHA 105 by electroporation. Oryza sativa ssp Japonica Rosa Marchetti was transformed as described previously[20]. Putatively, transformed plants (Hygromycin Resistant) were potted in a greenhouse

together with controls (untransformed, wild type rice). Total genomic DNA was isolated from leaves of putative transgenic and wild-type rice plants[21] and analyzed by PCR using specific primers for the human *ApoA-I* sequence. PCR reaction was performed by using the following cycle conditions: 94°Cx2'; 94°Cx45'', 55°Cx45'', 72°Cx45'' for 30 cycles; 72°Cx5'.

2.3 Rice milk production and ApoA-I protein quantification.

Rice seeds from wild type or transgenic plants were grinded in a fine powder. 100 gr of the obtained flour were liquefied at 90°C for 30 minutes in a solution of alpha-amylase (GAMALPHA SPEZIAL, Barentz) in protease-free aqueous medium (0,02% W/V NaCl) (100 ml Gamalpha spezial/t starch). An indirect competitive ELISA (IC-ELISA) was developed to detect ApoA-I protein in rice milk. Recombinant hApoA-I (Apolipoprotein A-I from human plasma, A0722, Sigma Aldrich) was coated onto micro-well plate overnight at 4°C. Then the plate was washed three times with 0,01 M PBS (pH 7) and blocked with 200 µl of 5% (W/V) BSA for 2h at 37°C. After washing the plate with 0,01 M PBS added with 0,05% (v/v) Tween 20 (PBST), 100 µl of the primary antibody (1:6000, goat polyclonal anti-ApoA1 antibody, ACRIS R1029P) solution were added to 100 µl of different dilution of rice milk. 100 µl of this mixture were added to each well and the plate was incubated at 37°C for 1 h. After washing the plate with PBST, 100 µl of anti-goat IgG-HRP antibody solution (1:10000) were added to each well and the plate was incubated at 37°C for 1 h. The plate was washed with PBST and a 50 µl of TMB were added to each well and the plate was incubated at 37°C for 15 min. In order to stop the reaction, 150 µl of 0,4 N hydrochloric acid (HCl) were added to each well, and absorbance was measured at 450nm by using an ELISA plate reader (BIORAD model 680). Each experiment has been performed in triplicates. In order to prepare a standard curve for the IC-ELISA, various parameters such as concentrations of coating antigen, primary and secondary antibodies, incubation time and temperatures were optimized[22]. Finally, on the basis of optimal conditions for IC-ELISA, the standard curve using recombinant hAPOAI protein was elaborated.

2.4 *In vivo* studies.

All experiments on animals were performed in accordance to the Italian Law and the European guidelines, following a protocol approved by the Institutional Committee for Animal Health (08/2014) and by the Ministry of Health (N. 202/2015-PR). For the tolerability study, 8-10 weeks old B6 male mice (Charles River, Calco, LC, Italy) were used. At day 0, mice were randomized in two groups (n=10 each group) and orally administered with WT or APOA-1M rice milk (10 ml/kg, 5d a week) for 3 weeks. At the end of the treatments, blood samples were collected for each animal. Hematological analyses were performed at the mouse facility of the University of Milano-Bicocca. For the efficacy study, 8-10 weeks old B6.129P2-*Apoe*^{tm1Unc}/J (*Apoe*$^{-/-}$) male mice were fed with Western Diet (Mucedola Srl, Settimo Milanese (MI), Italy) for 56 days *ad libitum*. After 56 days, mice were randomized in two groups (n=8 each group) and administered with APOA-1M or WT rice milk for 15 days by oral gavage. Western Diet was maintained for the whole period of the experiments. At the end of the treatments, animals were perfused and hearts, entire aortas and livers were harvested and processed for histology and immunohistochemistry analyses.

3. Results

3.1 APOA-1(Milano) muteins produced in seeds of genetically modified rice plants.

In order to overcome relevant purification issues that limited the potential use of APOA-1Milano (APOA-1M) proteins as a therapeutic agent, and since other groups clearly demonstrated that HDL mimetics can be efficiently delivered to disordered organisms by means of oral administration in the diet[23], we genetically engineered rice plants to express the full length APOA-1M in their seeds[24]. To this extent, the APOA-1M-expressing plasmid pPLT501 (Fig. 1A) was introduced in *A. tumefaciens* EHA105 strain and rice plants (Rosa Marchetti variety) were then transformed [20]. The presence of the transgene was verified by PCR on genomic DNA (gDNA) and the band of the expected size (732bp) corresponding to the APOA-1M amplicon was observed in gDNA samples corresponding to lanes 1 to 9 and lanes 11 and 12 (Fig. 1B).

To test if seeds from genetically modified rice plants did express APOA-1M protein properly, western blot analysis were performed on wild type and transgenic rice seed protein extracts. As shown in Figure 1C, in non-denaturing condition a band corresponding to 56 kDa was detected in transgenic rice lines corresponding to lanes 3, 6 and 7. A less intense band of 28 kDa was detected in the same samples, suggesting that these transgenic lines expressed APOA-1M protein primarily in the dimeric form. No signal was detected in wild type (WT) and transgenic lines 4 and 5 protein extracts (Fig. 1C). The genetically modified rice plant strain 7 was selected for further experiments. Western blotting carried out on seed pulps and seeds protein extract processed as 'rice milk' showed the same pattern as observed in transgenic and wild type protein extracts (Supplementary Figure 1A). No expression of APOA-1M was detected in leaves, stems, roots of the transgenic rice via western blotting analyses (data not shown), suggesting the tissue-specific expression of exogenous APOA-1M proteins. Interestingly, no degradation products were observed in any of the transformed sample, even 10 days after rice milk preparation (Supplementary Figure 1B), demonstrating a substantial protein stability over time. The amount of APOA-1M present in the rice seeds and in the rice milk was then evaluated by an ELISA test and it was estimated that rice seeds contained 49.12±0.27 μg of APOA-1M protein per gram of seeds and rice milk contained 33.20±0.97 μg of APOA-1M protein per gram of lyophilized product.

Taken together, these findings suggested that full length APOA-1M proteins can be expressed in genetically modified rice plants and that they maintain their ability to dimerize even after processing for production of seeds protein extracts (the 'APOA1M rice milk').

3.2 APOA1M rice milk administration was able to prevent macrophage activation and foam cell formation as well as to promote cholesterol efflux *in vitro*.

In order to test if APOA-1M proteins expressed in genetically modified rice plants were still able to exert anti-inflammatory activity, we evaluated the effects of APOA1M rice milk on the oxLDL-challenged THP-1 macrophages model *in vitro*. The APOA1M rice milk treatment was able to

prevent MCP-1 production by oxLDL-treated THP-1 cells more effectively than recombinant APOA-1 lipoprotein (Fig. 2A-B). Interestingly, MCP-1 production after oxLDL treatment was decreased proportionally to APOA-1M concentration (Fig 2C-D).

Since it is known that the main biological activity of APOA-1 protein is to facilitate the cholesterol metabolism by improving reverse cholesterol efflux [25], we then investigated if APOA-1M muteins retained the ability to reduce lipids accumulation in oxLDL-stimulated macrophages. Oil Red O assay of oxLDL-loaded THP-1 macrophages revealed a significant reduction of foam cell formation in APOA1M rice milk-treated cells as compared to controls (Fig. 2E-F). Finally, we next evaluated if the APOA-1M protein contained in the rice milk also retained the capacity to promote cholesterol efflux. APOA-1M rice milk, but not WT rice milk, significantly increased cholesterol efflux in THP-1 macrophages when delivered at 0.1 or 0.5 μg/ml of APOA-1M proteins (Fig. 2G).

3.3 APOA1M rice milk administration was well tolerated at the maximum tested dose.

Having demonstrated that the APOA-1Milano protein, delivered by means of transgenic rice seeds to macrophages, was effective in preventing macrophage activation and lipid accumulation *in vitro*, we then moved to test if these properties were maintained in an *in vivo* setting. First of all, the APOA1M-containing protein extract (APOA1M-MILK) was tested for tolerability in healthy mice, as rice milk administration to rodents has never been reported in literature before. To this extent, C57BL/6J mice were treated with WT rice milk (10 ml/Kg per day, 5 days a week) or APOA1M rice milk (10 ml/Kg per day which corresponds to 0.83 mg/Kg per day of APOA-1M muteins, 5 days a week) by oral gavage for 3 weeks. Animals from two experimental groups did not reveal signs of suffering. Hematologic values were in the range of normality (Supplementary Figure 2). Kidney and liver functions were not altered for any of the tested markers. Moreover, the total cholesterol did not undergo significant changes between the two groups and it was within normal range (Supplementary Figure 2). Taken together, these findings suggested that the delivery of APOA-1Milano molecules by means of genetically modified rice is not toxic and well tolerated by the healthy animals.

3.4 APOA1M rice milk reduced plaque extension and composition in *Apoe*$^{-/-}$ mice fed with high-cholesterol diet.

As rice milk administration was proved to be well tolerated, we assessed its therapeutic potential in an established model of early/intermediate atherosclerotic lesions. To this extent, *Apoe*$^{-/-}$ mice fed with WD for 8 weeks were treated with WT rice milk (10 ml/Kg per day, 5 days a week) or APOA-1M rice milk (10 ml/Kg per day which corresponds to 0.83 mg/Kg per day of APOA-1M muteins, 5 days a week) by oral gavage for 3 weeks. WD was maintained for the whole duration of experiments. As shown in figure 3, mice administered with APOA-1M rice milk developed atherosclerotic plaques (Fig 3A), positive to Oil Red O staining (Fig. 3B) and to macrophage infiltration (Fig. 3C), that had a significantly reduced extension as compared to wild type rice milk-treated mice (Fig. 3D). Moreover, Oil Red O staining revealed a consistent decrease in fatty acid accumulation, both in terms of area and intensity (Fig. 3E-F).

To assess the lipid deposition in aortas of atherosclerotic mice administered with WT (Ctrl) or APOA-1M (APO) rice milk, aorta *en face* was stained with Oil Red O (Fig. 3G). The treatment with APOA-1M rice milk significantly reduced the area of lipid deposition (Fig. 3H) and the concentration of tissue lipids (Fig. 3I) in aortic arch (AA) of WD-fed *Apoe*$^{-/-}$ mice as compared to WT rice milk-treated, WD-fed *Apoe*$^{-/-}$ mice. No significant reduction was observed in thoracic (TA) and abdominal (AbA) aorta. Furthermore, the area of lipid deposition in aorta *en face* positively correlated with the concentration of solubilized Oil Red O from entire aorta (Supplementary Fig. 3), demonstrating that the inhibition of the atherosclerotic plaque development was throughout the longitudinal section of the mouse aorta.

Taken together, these findings suggested that APOA-1M muteins, delivered via oral gavage in rice milk, were able to reduce atherosclerotic plaques size and lipids composition at the most atheroma-prone formation sites in the vascular system, even if the *Apoe*$^{-/-}$ mice were still exposed to WD diet.

3.5 APOA1M rice milk administration decreased macrophages infiltration in liver of *Apoe*$^{-/-}$ mice fed with high-cholesterol diet

Since it has been demonstrated that infusion of APOA-1M molecules has local as well as systemic anti-inflammatory effects[26], we then investigated if APOA-1M muteins, delivered by means of genetically modified rice seeds, could impact on the macrophages activation and recruitment in liver tissues. To verify this hypothesis, we measured CD68-positive cells in liver sections and we observed that the treatment with APOA-1M-rice milk significantly reduced the number of macrophages in liver as compared to the control group (35,36±1,74% and 29,67±1,20%, respectively, p=0.029, Figure 4A-B). On the other hands, the APOA-1M-rice milk treatment did not impact on hepatic fibrosis as revealed by Sirius Red staining on liver sections (Figure 4C-D).

Taken together, these findings suggested that APOA-1M muteins, delivered by means of genetically modified rice seeds, retained the anti-inflammatory properties as demonstrated by significant reduction of macrophages in liver of atherosclerotic mice.

4. Discussion

The potential of raising HDL-C as a therapeutic strategy for CVD rely on the role of these molecules in the reverse cholesterol transport process and are based on several evidences of efficacy on animal models [9,27] but it does not seem to be beneficial when translated in clinical settings. Among of the most promising HDL-targeted therapies, those involving infusion of HDL, either APOA-1, APOA-1Milano or Apo mimetics, reached interesting results in terms of atherosclerotic plaque reduction, but with relevant limitations in producing at large scale these drugs. Recently, the APO mimetics approach was demonstrated to be effective in animal models when orally delivered to the disordered organism[23,28,29].

We report here an innovative production and delivery system of full length APOA-1M molecules, without any need of purification, to be orally delivered to organisms by leaving them dissolved in their original biological context (seeds of rice plants). We take advantage of genetic modification of

rice plants to express the *ApoA-1M* coding sequence in their seeds, then deriving a protein extract from that seeds, the 'rice milk', that contains the APOA-1M molecules with intact anti-atherogenic and anti-inflammatory properties. SemBioSys Genetics Inc. previously reported a similar approach of genetic modification of plants to express in their seeds APOA-1Milano proteins[30], that was aimed to produce and purify the recombinant APOA-1Milano from the seeds of safflower. Our approach differs from this previous attempt in the fact that the APOA-1Milano protein produced in the seeds of transgenic rice does not need to be purified from the other proteins of the rice: it is produced within the delivery vehicle (the 'rice milk'). We firstly tested the efficacy of this "nutraceutic" approach *in vitro*, observing that APOA-1M rice milk administration to oxLDL-loaded macrophages caused a reduced expression of MCP-1, a key regulator of macrophages activation and chemo-attraction at the site of lesion and the subsequent formation of foamy cells, in a dose-dependent way. The APOA-1M rice milk also reduced MCP-1 expression and lipids accumulation of oxLDL-challenged macrophages as compared to recombinant ApoA-1 molecules. This was consistent with previously reported findings that the HDL_{Milano} variant is more effective in reducing macrophage activation and lipids accumulation *in vitro*[25,26]. Furthermore, the APOA-1M rice milk promoted cholesterol efflux *in vitro*, suggesting that the APOA-1Milano proteins present in rice milk also retained one of the human APOA-1 and APOA-1Milano key biological properties[31,32].

We then tested this novel delivery route of full length APOA-1M proteins for tolerability in healthy mice, since APOA-1M rice milk administration to rodent had never been reported in literature. The maximum dose tested of APOA-1M rice milk was based on the concentration of APOA-1M-containing lyophilized rice milk dissolved in water that could be orally administered to mice. The delivering, by oral gavage, 10 mL/Kg of a rice milk solution (2.5 g/mL) corresponded to the administration of 0.83 mg/Kg per day of APO-A1M to mice. This dosage was lower as compared to those studies delivering effective HDL_{Milano} via infusion (from 20 to 150 mg/Kg per each infusion) in atherosclerotic rabbit models[16,25], but on the same range of orally delivered effective APO mimetics (from 0.43 to 7.14 mg/Kg) in mice models[23]. The dosage of APOA-1M muteins that was

orally administered to mice models in our experimental settings allowed daily treatments without signs of suffering in healthy mice.

The anti-atherogenic effects of APOA-1M proteins, orally delivered by means of genetically modified rice seeds protein extract, were then evaluated in early/intermediate atherosclerotic lesion model[33,34]. To this extent, *Apoe*$^{-/-}$ mice were fed Western Diet for a total of 11 weeks, with the APOA-1M rice milk and control treatments starting at 8 weeks of WD feeding. Three weeks of APOA-1M rice milk treatment significantly reduced the plaque area at aortic sinus of WD-fed *Apoe*$^{-/-}$ mice as compared to WT rice milk-treated, WD-fed *Apoe*$^{-/-}$ mice (Fig. 3 A-B). Consistently with the reduction of lipids accumulation in oxLDL-loaded macrophages *in vitro* (Fig. 2C-D), the APOA-1M rice milk treatment also significantly reduced lipids accumulation at aortic sinus of WD-fed *Apoe*$^{-/-}$ mice (Fig. 3C-E). To better evaluate the anti-atherogenic properties of this delivery system, we then investigated the plaque area and lipid composition of entire aortas using *en face* analysis, widely recognized as a reliable method of murine atherosclerosis evaluation[35,36]. The atheroprotective effects of APOA-1M proteins, delivered by means of rice seeds extracts, were significant only at aortic arch and the restriction of the anti-atherosclerotic properties of tested system to the initial section of the aorta could be the result of pathophysiology of atherosclerosis development in this experimental model. The first atherosclerotic lesions in WD-fed *Apoe*$^{-/-}$ mice occur in aortic root and aortic arch and grow in size with age, while the lesions in other parts of vasculature, including descending aorta appear at later stages of atherosclerosis[33,37,38]. This explains the trend, not significant, to the reduction of the plaque area in thoracic and abdominal aorta of WD-fed *Apoe*$^{-/-}$ mice treated with APOA-1M rice milk. Notably, the significant reduction of atherosclerotic parameters (plaque area and lipids accumulation), observed in APOA-1M treated mice at aortic sinus and aortic arch, was achieved even if the *Apoe*$^{-/-}$ mice were still exposed to Western Diet during the therapeutic regimen, suggesting that APOA-1M proteins were able to abolish and even reduce the atherogenic effects of high fat diet. Taken together, these findings suggested that APOA-1M muteins, orally delivered as full length protein in rice milk at 0.83 mg/Kg per day, retained the anti-atherogenic

properties that other groups observed by infusion of higher concentration of HDL_{Milano}[16,25].

It has been demonstrated that infusion of HDL_{Milano} has also systemic anti-inflammatory effects[25,26]. Since it has been reported that 7 weeks of high fat diet is associated to hepatic inflammation in *Apoe*$^{-/-}$ mice[39], we wondered if the anti-inflammatory properties of APOA-1M could be retained in the oral delivery by means of rice seeds and we evaluated inflammation in liver of APOA-1M rice milk- or WT rice milk-treated *Apoe*$^{-/-}$ mice fed a WD. We demonstrated that the APOA-1M treatment slightly but significantly reduced hepatic CD68-positive cells (Fig. 4A-B), supporting the hypothesis that orally delivered APOA-1M proteins maintained anti-inflammatory properties in sites other than the vascular system. On the other hands, no reduction of hepatic fibrosis was observed (Fig. 4C-D) and this finding is consistent with the fact that fibrosis is a complex and multifactorial condition which requires a long-term therapeutic approach to resolve. Most of the works that demonstrated the ability of statins to significantly decrease liver fibrosis reported long-term treatments, both in human and in rodents[40-43]. Further investigations are needed to evaluate if a longer treatment period would also reduce a chronic and complex response such as fibrosis.

In conclusion, we reported an innovative production system, without any purification need, of full length APOA-1M proteins, by means of genetically engineered rice seeds. These APOA-1M muteins retained all the anti-atherogenic and anti-inflammatory properties when delivered to oxLDL-loaded macrophages in vitro and orally administered to atherosclerotic *Apoe*$^{-/-}$ mice. Further investigations are needed to identify the minimum effective dose and the confirmation of the oral bioavailability of APOA-1M muteins administered to disordered organisms. Nevertheless, the positive results of our proof-of-concept study, in which the APOA-1M treatment was delivered to mice still exposed to risk factors, could expand the therapeutic options in terms of prevention for those patients that are refractory to change habits or with a continuous exposure to risk factors. Overall, the nutraceutical approach described in the present work may pave the way for a new avenue of therapeutic products in atherosclerosis and possibly other diseases, by virtue of a safe and cost-effective route of administration which is able to deliver optimal dosages of active principle.

Figure Captions

Figure 1. Production and characterization of APOA-1(Milano) muteins-expressing genetically modified rice plants. (A) Schematic representation of the pPLT501 plasmid expressing the *APOA-1M* gene under the control of rice prolamine promoter (ProI). (B) PCR on gDNA of different APOA-1M genetically modified rice plants lines (1-12). Transgenic plants (1-9 and 11-12) showed the amplification of the expected band (732 bp). M: molecular weight ladder; C-: PCR on gDNA from the untransformed rice (Rosa Marchetti), as negative control; C+: positive control. (C) APOA-1 protein western blot analyses on total protein extracts from transgenic rice lines (3-7) in non-denaturing condition. The genetically modified rice lines 3, 6, and 7 showed the expression of APOA-1M protein primarily in the dimeric form (58 kDa band). No signal was detected in the wild type rice seed extract (WT) and in transgenic rice lines 4 and 5. C+: protein extract from human serum carrying the APOA-1M mutation; C-: protein extract from human serum carrying the wild type APOA-1 gene.

Figure 2. APOA-1M in rice milk is effective in preventing macrophages activation and lipids accumulation following exposure to oxLDL and in promoting cholesterol efflux. (A) Immunoblotting analysis for MCP-1 on THP-1 macrophages lysates exposed or not to oxLDL and/or WT or APOA-1M rice milk and (B) relative quantification of immunoblotting bands. MCP-1 expression was significantly induced by oxLDL administration. APOA1-M and not APOA1-R was able to inhibit MCP-1 expression by THP-1 macrophages. * $p<0.05$. Error bars represent SEM. (C) Immunoblotting analysis for MCP-1 on THP-1 macrophages exposed to decreasing concentrations of APOA-1M rice milk. (D) MCP-1 expression was inversely proportional to APOA1-M concentration in rice milk. (E-F) Rice milk containing APOA-1Milano inhibited lipids accumulation in THP-1 macrophages. Representative pictures of Oil Red O staining of 3 independent experiments are illustrated in panel E. (F) Oil Red O staining was significantly reduced by the administration of rice milk containing APOA-1 Milano. *$p<0.05$. Error bars represent SEM.

(G) Cholesterol efflux was efficiently promoted in THP-1 macrophages by rice milk containing APOA-1M at a concentration of 0.1 and 0.5 μg/ml. No effects were observed in THP-1 macrophages treated with the same amount of WT rice milk. *p<0.05, **p<0.01, ***p<0.001. Error bars represent SEM.

NT: control cells that did not receive nor WT nor APOA-1M rice milk. Pictures in (A) and (C) are representative of 5 independent experiments. APOA1-M concentration in rice milk and APOA1-R concentration were 2 μg/ml where not indicated.

Figure 3. APOA-1M rice milk reduces plaque extension and composition in *Apoe*$^{-/-}$ mice fed with high-cholesterol diet. Representative pictures of Hematoxylin and Eosin (A), Oil Red O staining (B) and CD68-positive macrophage infiltration (C) of hearts from high-fat diet fed *Apoe*$^{-/-}$ mice, treated with WT (n=6) or APOA-1M (n=8) rice milk for 15d, 5d/week. Bars represent 500 μm in (A) and (B) and 100 μm in (C), arrowheads indicate atherosclerotic plaques. APOA-1M rice milk was able to significantly reduce Plaque Area (D), Oil Red O positive area (E) and Oil Red O intensity (F).; **p<0.01. Error bars represent SEM. (G) Representative images of the aorta *en face* of *Apoe*$^{-/-}$ mice on Western Diet administered with WT (Ctrl, n=6) or APOA-1M (APO, n=8) rice milk for 15d, 5d/week by oral gavage, stained by Oil Red O (red), area pre-processed for plaque selection (green) and final plaque selection area (green with blue borders) using ImageJ's thresholding tool. Bar = 5 mm. (H) The quantification of aortic lipid lesions as a percentage [%] of individual aortic section area using ImageJ's thresholding tool. (I) The quantification of aortic lipid lesions as a concentration of solubilized Oil Red O per mm^2 of individual aortic sections. Error bars represent mean ± SEM (H, I). **p<0.01 by two-way ANOVA followed by Sidak post hoc test (H, I).

Figure 4. APOA1M rice milk administration slightly decreased inflammation in liver of *Apoe*$^{-/-}$ mice fed with high-cholesterol diet. (A) Representative pictures of CD68 immunohistochemistry

on liver sections of WT milk (left) or APOA-1M milk (right) treated mice. Bars represent 100 μm. (B) APOA-1M rice milk significantly reduced the number of CD68-positive cells in liver of *Apoe*$^{-/-}$ mice fed a high cholesterol diet. At least 8 different microscope fields for each liver section were analyzed. * $p<0.05$. Error bars represent SEM. (C) Representative pictures of Sirius Red staining (extracellular matrix deposition and accumulation) on liver sections of WT milk (left) or APOA-1M milk (right) treated mice. Bars represent 100 μm. (D) APOA-1M rice milk did not alter the fibrotic deposition in liver of *Apoe*$^{-/-}$ mice fed a high cholesterol diet. MRI fibrosis tool for ImageJ (http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Fibrosis_Tool) was used to measure the relative area of sirius red stained fibrosis.

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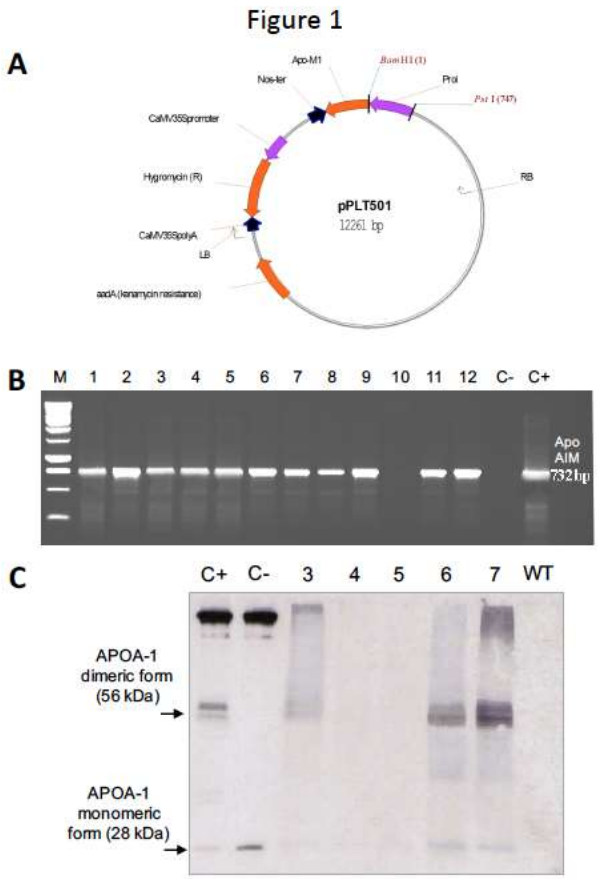
Figure 1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BamHI (1)
Nos-ter
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CaMV35Spromoter
Hygromycin (R)
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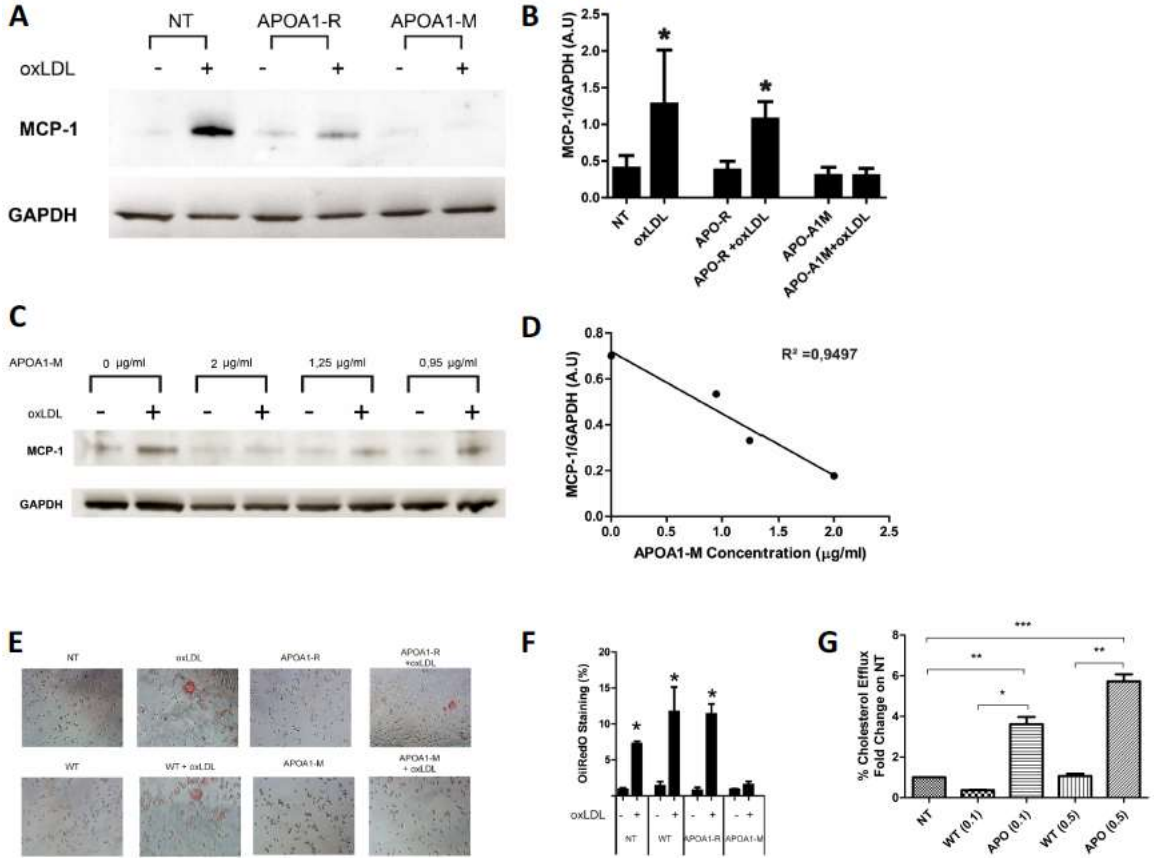
12261 bp
CaMV35SpolyA
LB
aadA (kanamycin resistance)
B
M 1 2 3 4 5 6 7 8 9 10 11 12 C- C+
Apo AIM 732 bp
C
C+ C- 3 4 5 6 7 WT
APOA-1 dimeric form (56 kDa)
APOA-1 monomeric form (28 kDa)

1

2

Figure 2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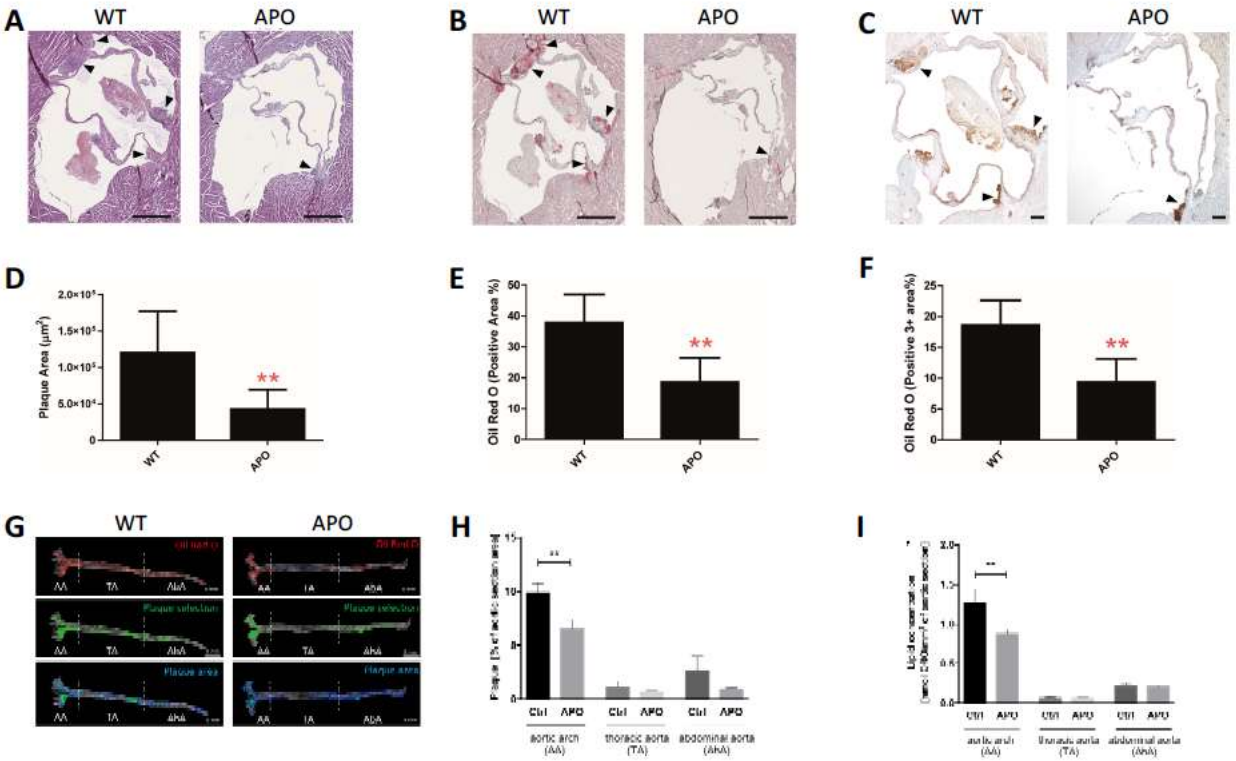
NT
APOA1-R
APOA1-M
oxLDL
MCP-1
GAPDH
B
MCP-1/GAPDH (A.U)
NT
oxLDL
APO-R
APO-R +oxLDL
APO-A1M
APO-A1M+oxLDL
C
APOA1-M
0 µg/ml
2 µg/ml
1,25 µg/ml
0,95 µg/ml
oxLDL
MCP-1
GAPDH
D
R² =0,9497
MCP-1/GAPDH (A.U)
APOA1-M Concentration (µg/ml)
E
NT
oxLDL
APOA1-R
APOA1-R +oxLDL
WT
WT + oxLDL
APOA1-M
APOA1-M + oxLDL
F
OilRedO Staining (%)
oxLDL
NT
WT
APOA1-R
APOA1-M
G
% Cholesterol Efflux Fold Change on NT
NT
WT (0.1)
APO (0.1)
WT (0.5)
APO (0.5)

Figure 3


A
WT
APO
B
WT
APO
C
WT
APO
D
Plaque Area (μm²)
2.0×10⁵
1.5×10⁵
1.0×10⁵
5.0×10⁴
0
**
WT
APO
E
Oil Red O (Positive Area %)
50
40
30
20
10
0
**
WT
APO
F
Oil Red O (Positive 3+ area%)
25
20
15
10
5
0
**
WT
APO
G
WT
APO
AA
TA
AbA
H
**
Ctrl
APO
aortic arch (AA)
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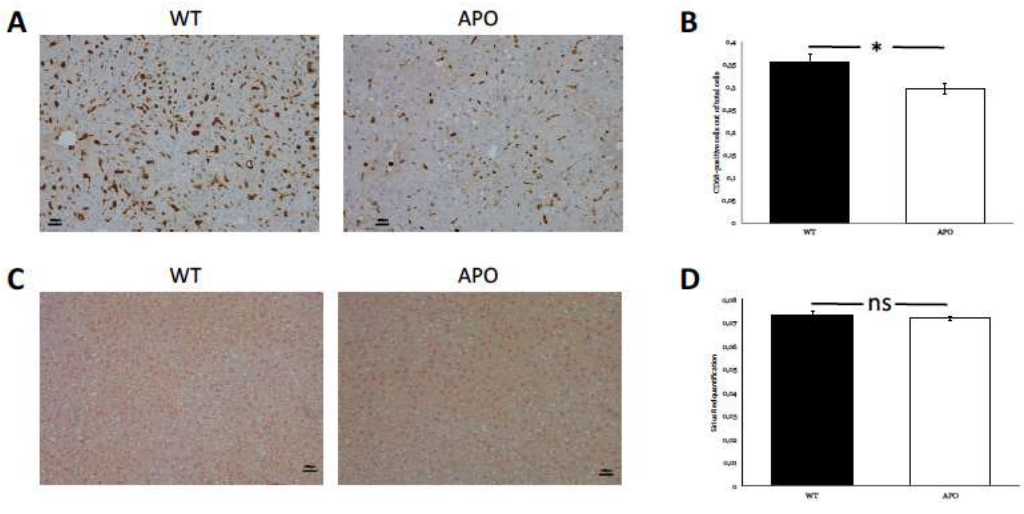
abdominal aorta (AbA)
I
**
Ctrl
APO

1

2

Figure 4



1