Effects of D- Mannose on atherosclerosis

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Abstract (English)

Background: D-mannose, a C−2 epimer of glucose, is an important monosaccharide for protein glycosylation that is widely distributed in body fluids and tissues. Interestingly, D-mannose has been shown to prevent obesity in C57BL/6 young mice fed a high fat diet (HFD) through regulation of gut microbiota. Furthermore, several studies have shown that mannose exhibits potent anti-inflammatory properties. What remains undefined is whether D-mannose regulates atherosclerosis, a chronic inflammatory disease of the arteries initiated and driven by sub-endothelial oxidised lipid accumulation. We hypothesize that D-mannose supplementation would alleviate the pro- atherogenic effects of HFD by regulating the gut microbiota and inflammation.

Methods & results: ApoE knockout mice, an atheroprone mouse model, received a HFD during 9 weeks. Concurrently, one group of mice received 0 (control), 5 or 20% D-mannose administered orally and another group received intraperitoneal (IP) injections of 5g/kg D-mannose 5 times a week or PBS as a control. Increases in body weight and plasma lipid levels were equivalent in all mannose-supplemented groups as compared with Ctr groups. Plasma mannose levels increased by 2.4 and 1.4 times in mice orally supplemented with 5 and 20% D-mannose respectively (89±15 (5%), 51±16 (20%) vs 37±5ng/ml) ($P<0.05$). Gut microbiota 16S sequencing analysis indicated a significant increase in Firmicutes/Bacteriodetes ratio in mice fed a HFD (HFD Ctr) compared with Chow diet (Chow Ctr), this increase was prevented in mice fed a HFD supplemented with 20% mannose (HFD+20% mannose) (4.8±0.9 (HFD Ctr), 2.8±0.4 (20%) vs 1.7±0.3ng/ml). This was associated with a decrease in intestinal F4/80+ macrophage number. Oral D-mannose supplementation at 5 and 20% also reduced by twice the expression of lipopolysaccharide receptor, Toll-like receptor 4 on macrophages in the lamina propria of small intestine compared with Ctr, $P<0.05$ and $P<0.01$. This suggests an anti-inflammatory effect of mannose in the gut. Accordingly, plasma LPS levels were increased by HFD compared with chow (11 vs 6 EU) and 20% mannose supplementation prevented this increase. Flow cytometry performed on blood and peritoneal cavity cells revealed that proportions of blood pro-inflammatory $Ly6C^{HI}$ monocytes as well as thioglycolate-elicited small peritoneal macrophages (SPM) were reduced significantly by 40 and 34% respectively, in 20% oral D-mannose mice vs 0% controls. In addition, bone marrow progenitor cells as well as Ly6C+ monocytes were evaluated by flow cytometry. c-Kit+sca1- progenitors and $Ly6C^{LO/HI}$-expressing monocyte ratio

was significantly decreased by 22 and 42% in oral 20% mannose-treated mice vs 0% controls. Importantly, atherosclerotic plaque size as determined by Oil red O staining, was significantly reduced in mice receiving 5 and 20% D-mannose in drinking water compared with 0% Ctr (aortic sinus: 0.23±0.03 (5%), 0.20±0.05 (20%) vs 0.42±0.04mm^2, brachiocephalic artery: 0.05±0.01(5%), 0.05±0.02 (20%) vs 0.08±0.01mm^2; $P<0.05$. Plaque stability index was evaluated by plaque α-smooth muscle actin and necrotic core ratio, which was increased by 4 times in 20% oral mannose treated mice as compared with control group, $P<0.05$. However, CD68+ macrophages within plaques were not different between oral mannose and control groups. Interestingly, no changes in plaque size were observed between mannose supplemented by IP and control (PBS) groups in the aortic sinus and the brachiocephalic artery (BCA). These results suggest the possible involvement of gut microbiota in the observed athero-protective effect of mannose.

Conclusion: Taking together, oral D-mannose supplementation reduces atherosclerotic lesions in mice and increases plaque stability. These protective effects of D-mannose could possibly occur through inhibition of pro-inflammatory monocytes/macrophages following regulation of gut microbiota composition.

Résumé (français)

Contexte et hypothèse: D-mannose (mannose henceforth) , un C-2 épimère du glucose, est un monosaccharide important pour la glycosylation des protéines et qui est très présent dans les fluides corporels et les tissus. Il a été démontré que mannose prévient l'obésité chez les jeunes souris C57BL/6 nourries avec un régime riche en gras par la régulation du microbiote intestinal. De plus, plusieurs études ont montré que le mannose a de puissantes propriétés anti-inflammatoires. Ce qui reste inconnu est si le mannose régule l'athérosclérose, une maladie inflammatoire chronique des artères initiée et entraînée par l'accumulation de lipides oxydés sous la couche des cellules endothéliales. Nous émettons l'hypothèse que la supplémentation en mannose atténuerait les effets pro-athérogéniques du régime riche en gras en régulant le microbiote intestinal et l'inflammation.

Méthodes et résultats : Des souris ApoE$^{-/-}$, qui est un modèle athéroprone, ont reçu un régime riche en gras pendant 9 semaines. Un groupe de souris a reçu 0 (Ctr), 2, 5, 10 ou 20% de mannose administré par voie orale et un autre groupe a reçu des injections intrapéritonéales (IP) de 5g/kg de mannose 5 fois par semaine ou du PBS comme control. Les augmentations du poids corporel et des taux de lipides plasmatiques étaient équivalentes dans tous les groupes expérimentaux supplémentés en mannose par rapport aux groupes Ctr. Les taux plasmatiques de mannose était augmenté de 2.4 et 1.4 fois chez les souris supplémentées par voie orale avec 5 et 20% de mannose respectivement (89±15 (5%), 51±16 (20%) vs 37±5 ng/ml) ($P<0.05$). L'analyse du séquençage 16S du microbiote intestinal a indiqué une augmentation significative du rapport des bactéries Firmicutes/Bacteriodetes chez les souris sous régime riche en gras par rapport au régime normal, cette augmentation a été prévenue chez les souris sous régime riche additionné de 20% de mannose (HFD+20% mannose) (4.8±0.9 (HFD Ctr), 2.8±0.4 (20 %) vs 1.7±0.3). Ces résultats étaient associés à une diminution du nombre de macrophages intestinaux F4/80+. La supplémentation orale en mannose à 5 et 20% a également réduit de 50% l'expression du récepteur de lipopolysaccharide (LPS), TLR-4 sur les macrophages de la lamina propria de l'intestin grêle par rapport au groupe Ctr, $P<0.05$ et $P<0.01$. Ces résultats suggèrent un effet anti-inflammatoire du mannose dans l'intestin. En conséquence, le taux plasmatique de LPS était augmenté par le régime riche en gras par rapport au régime normal (11 vs 6 EU) et une supplémentation en 20% mannose a empêché cette augmentation. La cytométrie de flux réalisée sur les cellules sanguine et

de la cavité péritonéale a montré une réduction significative des proportions de monocytes pro-inflammatoires Ly6C^{HI} ainsi que des petits macrophages péritonéaux (SPM) induits par le thioglycolate de 40 et 34% respectivement, chez les souris avec 20% de mannose vs 0%. Les cellules progénitrices de la moelle osseuse ainsi que les monocytes Ly6C+ ont également été évalués par cytométrie de flux. Les progénitrices (c-Kit+Sca1-) et le ratio des monocytes Ly6C$^{LO/HI}$ étaient significativement diminués de 22 et 42% chez les souris traitées avec 20% de mannose par voie orale par rapport aux Ctr. La taille de la plaque d'athérosclérose, déterminée par coloration à l'huile rouge, était significativement réduite chez les souris recevant 5 et 20% de mannose oralement par rapport aux Ctr (sinus aortique : 0.23±0.03 (5%), 0.20±0.05 (20%) vs 0.42±0.04mm^2, artère brachiocéphalique : 0.05±0.01 (5%), 0.05±0.02 (20%) vs 0.08±0.01mm^2, $P<0.05$. L'indice de stabilité de la plaque a été évalué par le ratio de α-smooth muscle actin de la plaque et les cores nécrotiques. Le ratio était quatre fois plus grand chez les souris traitées au mannose à 20% par rapport au groupe control, $P <0.05$. Cependant, les macrophages CD68 + de la plaque etaient identiques entre les groupes mannose et Ctr. Aucune modification dans la taille de la plaque n'a été observée entre les groupes mannose et contrôles (PBS) dans le sinus aortique et l'artère brachiocéphalique. Ce résultat suggère l'implication du microbiote intestinal dans l'effet athéro-protecteur observé du mannose.

Conclusion: La supplémentation orale en mannose réduit les lésions athérosclérotiques chez la souris et augmente la stabilité de la plaque. Ces effets protecteurs du mannose pourraient possiblement être attribués à l'inhibition des monocytes/macrophages pro-inflammatoires suite à une régulation de la composition du microbiote intestinal.

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Contribution of authors

I (Jonathan O'Connor) reviewed and synthesized the relevant literature in order to put this project into a meaningful context. I wrote the present thesis in its entirety, receiving some feedback and corrections from my supervisor, Dr. Stephanie Lehoux and my research associate Talin Ebrahimian. Finally, I was partly or fully involved in carrying out all the experiments and analyses described below, with training and guidance from several members of my lab.

Dr. Stephanie Lehoux is the lab's principal investigator and my thesis project supervisor. She provided me with feedback, guidance, and support throughout the entire project. Dr. Lehoux generated the hypothesis underlying this project and designed the experiments to be conducted. Dr. France Dierick, a postdoctoral fellow in our lab, trained me to perform the mouse dissections and other *in vivo* techniques. Dr. Dierick taught me how to perform whole tissue preparation, cryosectioning, and immunofluorescence staining of the tissue sections. Finally, Dr. Talin Ebrahimian, helped me greatly in conducting the flow cytometry experiments and analyses. She provided significant feedback and corrections in translating the abstract of this thesis to French.

Dr. Ebrahimian provided thorough guidance through my thesis project. Most notably, she taught me how to extract bone marrow cells from mice, and to perform migration and stimulation experiments with these cells. She also taught me how to extract macrophages from the mouse peritoneal cavity. Dr. Ebrahimian designed the panel of fluorescent antibodies used in this thesis. Maria Kotsiopriftis taught me how to perform western blots and DNA extraction.

List of Figures and Tables

Introduction

Atherosclerosis is a chronic inflammatory disease that plays an essential role in the pathogenesis of cardiovascular disease (CVD), a leading cause of death worldwide. Lipid lowering statins are currently used as a drug therapy to reduce cardiovascular morbidity and mortality. However, many patients are still at risk for experiencing relapses in heart attacks and other CVD events. Therefore, it is important to complement the therapy with lifestyle changes, a key in reducing cardiovascular events. Atherosclerosis develops from different modifiable and non-modifiable risk factors, such as obesity and diabetes mellitus directly influenced by our diet. D-mannose (mannose henceforth), a C-2 epimer of glucose, is a monosaccharide approximately a hundred times less abundant than glucose in human blood. It plays important roles in the glycosylation of certain human native proteins and previous studies have demonstrated that mannose, from now on called mannose, has immunoregulatory effects and prevents mouse weight gain under high fat diet (HFD) conditions through regulation of gut microbiota. Gut microbiota is the collection of bacteria that inhabit in the gastrointestinal tract producing a diverse ecosystem about 10^{14} microorganisms and the homeostasis of gut microbiota is critical for maintaining human health. Gut dysbiosis has been shown to contribute to the development of various diseases including CVD. We hypothesize that mannose supplementation to HFD in mouse could through regulation of the gut microbiota inhibit inflammation and prevent atherosclerosis.

List of abbreviations

ABC	ATP-binding cassette transporters
ApoB	Apolipoprotein B
BA	bile acid
BCA	Brachiocephalic artery
CAM	Cell adhesion molecules
CVD	Cardiovascular disease
EC	Endothelial cells
ECM	Extracellular matrix
EndMT	Endothelial-to-mesenchymal transition
FC	Foam cell
HDL	High-density lipoprotein
HFD	High Fat Diet
LDL	Low-density lipoprotein
LPM	Large peritoneal macrophages
LPS	Lipopolysaccharide
NC	Necrotic core
OxLDL	Oxidised LDL
PBS	Phosphate-buffered saline
PerC	Peritoneal cavity
ROS	Reactive oxygen species
SCFA	Short chain fatty acid
SPM	Small peritoneal macrophages
SR	Scavenger receptor
VCAM	Vascular cell adhesion molecule
VSMC	Vascular smooth muscle cell

1. Literature review

1.1 Atherosclerosis

1.1.1 Overview of atherosclerosis

Atherosclerosis is a chronic inflammatory disease in which the gradual accumulation of excess circulating lipids and immune cells in the arterial wall produces lesions, called plaques, that progressively narrow the arterial lumen. While these plaques can remain clinically silent throughout the first several decades of life, their continual growth can eventually lead to the partial or complete blockage of the affected arterial segments, impairing circulation to downstream tissues. More importantly, they may become increasingly unstable and susceptible to rupturing over time; when this occurs, the release of plaque contents into the circulation triggers a rapid formation of blood clots, which can obstruct blood flow either immediately at the site of formation or elsewhere in the circulation following their dislodgement. This results in tissue death and is the main cause of life-threatening cardiovascular diseases such as heart attack and stroke [1, 2].

1.1.2 Biomechanical basis of atherosclerosis

Atherosclerotic lesions begin to develop in childhood where accumulation of the lipids in the wall and foam cells have been detected in infants as early as 6 months [3-7]. They tend to form specifically at vascular transition points such as bifurcations, branch points, and curvatures, where the vessel deviates from a straight line and imposes an abrupt change the direction flow and generating oscillatory shear of the region [8-16]. As shown in Figure 1 [17], the flow patterns exert oscillatory shear stress. Most regions of adult arteries are exposed to high shear stress, which is associated with protection from atherosclerotic plaque development [18, 19]. Nevertheless, lesions can still form here in severe cases [20, 21]. Branches and bends are exposed to low and oscillatory shear stress, which causes inappropriate activation of developmental signalling pathways, leading to

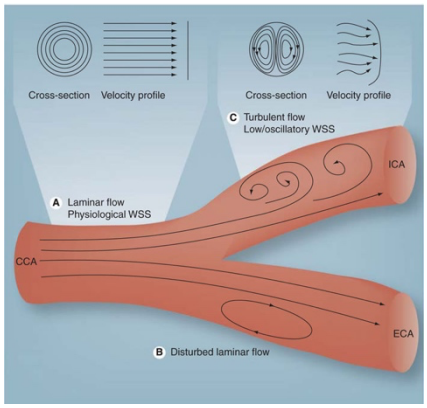


Figure 1. The disruption of regular, laminar blood flow at vascular transition points serves as the biomechanical basis for the initial formation of atherosclerotic plaques.

increased inflammation and vascular permeability, which are the hallmarks of early atherogenesis [22-25].

1.1.3 ApoE knockout mouse model

Despite its limitations, the mouse remains the favored species for atherosclerosis investigation due to the ease of breeding, low cost of maintenance, and relatively short period for developing atherosclerosis. The two most frequently used models of mouse atherosclerosis are the ApoE E−/− model and the LDLR−/− model. They differ in their dietary needs for developing atherosclerosis. The ApoE −/− model is perhaps the most widely used. The advantage of the ApoE −/− model is that complex vascular lesions develop in animals fed the normal low fat chow diet, even when plasma cholesterol levels are between 300–500 mg/dl. These lesions are comparable to human lesions. The rate of atherogenesis can be notably accelerated by the feeding of a HFD, resulting in significant increases in plasma lipid levels (>1,000 mg/dl). Pathological analysis of the evolution of atherosclerosis in ApoE −/− mice fed either chow or HFD represents the best systematic analysis of lesion development in mice [26]. Lesions developed in the aortic root, the brachiocephalic artery and other branches of the aorta, as well as the pulmonary and carotid arteries. Despite the widespread use of the ApoE −/− model, it has several disadvantages. Plasma cholesterol is mostly carried on lipoprotein remnants rather than the LDL, which is the most frequent “offending” particle in human atherosclerosis. The hyperlipoproteinemia of ApoE −/− mice is largely attributable to the absence of the lipoprotein ligand for the major cell surface receptors responsible for high affinity plasma lipoprotein clearance. However, ApoE has other functions affecting macrophage biology, immune function and adipose tissue biology [27], each of which could have an impact on atherosclerosis independent of plasma lipid levels.

1.1.4 Initiation of atherosclerosis

1.1.4.1 Role of lipids in atherosclerotic plaque formation

Early in life, long before the onset of any pathological changes, the intima (the most inner layer of arteries, which includes the endothelium along with the underlying extracellular matrix (ECM) and smooth muscle cells (SMCs)) undergoes an adaptive thickening in the aforementioned susceptible regions of low shear stress, as a result of the non-uniform hemodynamic conditions

that occur there [28-30]. Physiologically, this helps to maintain vascular homeostasis by stabilizing blood flow velocity and preserving structural integrity [31-35]. These thickened regions tend to collect LDLs, one of the main lipid carriers in the systemic circulation and a major component of the atherosclerotic plaques. Although there are low levels of LDL found in the healthy intima [36-38], abundant LDL accumulation is considered the initiating event in atherogenesis [28, 39-42]. In addition to increased endothelial permeability [43-50], these vulnerable areas have increased lipoprotein retention [40, 42, 50] through ionic interactions between the positively-charged apolipoprotein B (ApoB, the major protein scaffold component of LDL) and the negatively-charged proteoglycans in the intimal ECM [48]. It is now well understood that the progression of atherosclerosis does not simply involve a passive accumulation of lipids, but rather a complex inflammatory response in which various immune cells, particularly monocytes, are recruited in an attempt to remove LDL from the plaque [51, 52]. These phagocytes take up the LDL via cell surface LDL receptors (LDLR), a process that is regulated by negative feedback. The increase in intracellular cholesterol is detected by transcription factors called sterol regulatory element-binding proteins, which downregulates LDLR at the cell surface to prevent excess LDL uptake [53-57]. In addition, high intracellular cholesterol levels upregulate ATP-binding cassette transporters, ABCA1 and ABCG1 [58] . These proteins load cholesterol into high-density lipoprotein (HDL) [59-61], the "good cholesterol" carrier which recirculates excess cholesterol from tissues back to the liver, where it can be eliminated through bile excretion.

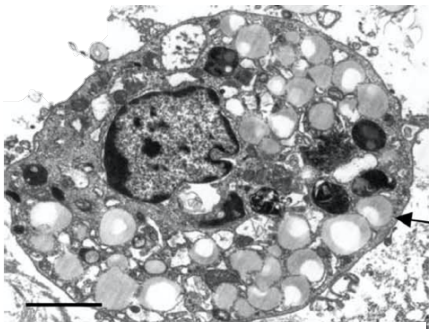


Figure 2. Foam cell. Transmission electron microscopy in the human arterial intima, the majority of monocytes differentiate and transform into foam cells.

LDL undergoes modifications, especially oxidation (oxLDL), in the intima [62], and is taken up through scavenger receptors (SRs) such as SR-A and SR-B (CD36) or lectin-like oxLDL receptor-1 (LOX-1) [63, 64]. Unlike LDLR, SR-mediated uptake of modified LDL is not regulated by negative feedback, thereby permitting substantial intracellular LDL accumulation and macrophage foam cell (FC) formation (Figure 2[65]) [53, 66-69].

1.1.4.2 Cells involved in plaque formation

- Endothelial cells

Endothelial cells (ECs) play an important role in sensing the biomechanical factors that predispose certain regions of the arteries to atherosclerosis according to the changes in the shear stress [70, 71]. These cells can detect through mechanoreceptors and the cytoskeleton these changes in the stress influence the gene expression in ECs, allowing them to adaptively respond to hemodynamic changes [72-75]. Some genes described are bone morphogenetic protein 4, transforming growth factor-β, components of the WNT signalling pathway, genes related to endothelial-to-mesenchymal transition (EndMT), the transcription factor hypoxia-inducible factor 1α and its targets vascular endothelial growth factor and glycolysis-related genes and angiopoietin receptors [76-80]. Cultured ECs exposed to low shear stress have increased surface expression of cell adhesion molecules (CAMs) [81-83], which permit the attachment of leukocytes. CAMS are also known to be induced under pro-inflammatory conditions [84-87] such as during atherosclerosis. *In vivo*, there is increased permeability at the endothelium, accumulation and oxidation of lipoproteins (LDL), expression of CAMs, and recruitment of monocytes [30, 88-91]. All these events promote the formation and progression of atherosclerotic lesions [31, 92-97].

- Monocytes

First, monocytes which are the circulating precursors of macrophages, begin rolling on the endothelial surface. They could have detrimental or beneficial functions, depending on their subsets that display heterogeneity in both mouse and human. In mouse $Ly6C^{HI}$ ($Gr1^{HI}CCR2+CX3CR1^{LO}$) are considered as pro inflammatory monocytes subsets [98, 99]. In contrast, $Ly6C^{LO}$ ($Gr1^{LO}CCR2-CX3CR1^{HI}$) monocytes are anti-inflammatory determined as a patrolling MC subset and are involved in tissue repair [100-103]. Pro inflammatory $Ly6c^{high}$ monocytes in mice interact with P-selectin glycoprotein ligand 1 and endothelial P- and E-selectins [104-106]. Rolling is followed by firm adhesion, through vascular cell adhesion molecule–1 (VCAM-1) and intracellular cell adhesion molecules (ICAM-1), respectively [90, 107]. Next, they cross the endothelial barrier into the underlying intimal space under the influence of chemokines

such as monocytes chemoattractant protein-1 (MCP-1, also known as CCL2), CX3CL1, and CCL5 [108-111]. Monocytes make up most of the infiltrating cells and differentiate into macrophages within the atherosclerotic plaque. In the early stages of atherogenesis, oxLDL induces the expression of MCP-1 and CX3CL1 by vascular smooth muscle cells and endothelial cells. MCP-1 is found in the vessel wall where macrophage-rich areas bordering the lipid core, as well as in endothelial and VSMCs in human, mouse, and rabbit atherosclerotic lesions and promotes the transendothelial migration of CCR2-expressing monocytes [112]. It has been identified directly on foam cells and influences the growth of other cell types within the atherosclerotic lesion [113, 114]. CX3CL1 is the only known chemokine able to act both as a chemoattractant and as an efficient adhesion molecule through CX3CR1 G-protein-linked receptor [115, 116]. It is expressed on monocytes, natural killer cells, T cells, and smooth muscle cells, where it mediates migration, adhesion and proliferation [117]. Deletion of CX3CL1 in CCR2-deficient mice dramatically reduced the accumulation of macrophages in the arterial wall and the subsequent development of atherosclerosis [118]. CCL5 through interaction with its receptor CCR1, is also considered an early pathway leading to accumulation of rolling monocytes to stimulated endothelial cells. Also, CCL5 induces SMC proliferation and could have a role in the plaque stability [119]. Combined inhibition of CCL2, CX3CR1, and CCR5 in hypercholesterolemic, atherosclerosis-susceptible apolipoprotein E-deficient (ApoE) mice leads to abrogation of bone marrow monocytosis which are critical mediators of atherosclerosis onset, and to additive reduction in circulating monocytes despite persistent hypercholesterolemia [109].

- Macrophages

Macrophages are the major immune cells in atherosclerotic lesions. They are derived from myeloid progenitor cells in the bone marrow[120]. They have developed remarkable plasticity, notably, the ability to promote inflammation when needed and to turn the inflammatory response off when no longer needed[121]. In experimental systems, the classical inflammatory macrophage phenotype has been termed M1 and is often induced by incubating macrophages *in vitro* with a combination of interferon-γ (IFN-γ) and the toll-like receptor 4 (TLR4) ligand, lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria. These stimuli initiate a strong inflammatory response, which includes production of pro-inflammatory cytokines, such as

interleukin-1β (IL-1β), IL-12 and tumor necrosis factor-α (TNF-α), chemokines, such as MCP-1 (CCL2) to attract more monocytes, and inducible nitric oxide synthase (iNOS)[122, 123]. The M2 phenotype is mainly induced by IL-4 and IL-13 cytokines, both of which are able to inhibit the M1 phenotype and prompt the macrophages to produce pro-resolving molecules, such as IL-10 and TGF-β. M2 macrophages are involved in tissue remodeling and repair [124]. Activated ECs release macrophage colony-stimulating factor (M-CSF), which promotes monocyte differentiation into macrophages [1, 98]. Macrophages uptake oxidised lipids and turn into foam cells (Fig. 3).

Recently, another macrophage subtype has been described in atherosclerotic lesions, called aorta intima resident macrophage (Mac^{AIR}). These macrophages infiltrate the artery around birth and arise from monocyte progenitors, thereafter adopting self-renewing capacity and expressing CD11c and MHC II. Mac^{AIR} were observed to accumulate lipid in nascent arteries and promote foam cell formation within the plaque [125]. These cells are thought to differentiate into the first foamy cells within in the arterial intima prior to the recruitment of monocytes. Prolonged hypercholesterolemia leads to the complete loss of the resident-derived foamy cells, as they are ultimately replaced entirely by recruited blood monocytes [126].

Macrophages are also recruited to the peritoneal cavity (PerC). There are 2 main subsets found in adult mice, based on their size and expression of F4/80 marker. Small peritoneal macrophages (SPM) and large peritoneal macrophages (LPM). Approximately 90% of PerC macrophages are LPMs, are long-lived and self- renewing population derived from the yolk sac progenitors under basal unstimulated conditions. They disappear rapidly from the PerC following LPS or thioglycolate stimulation. These cells express high levels of canonical macrophage surface markers, CD11b and F4/80 [127]. SPMs are short-lived and replenished from circulating monocytes infiltrating the peritoneum in inflammatory conditions. They express substantially lower levels of CD11b and F4/80 but high levels of MHC-II, which is not expressed on LPMs. SPM, which predominates in PerC after LPS or thioglycolate stimulation, does not derive from LPM. Both subsets show clear phagocytic activity and produce nitric oxide (NO) in response to LPS stimulation *in vivo* [103, 127-132].

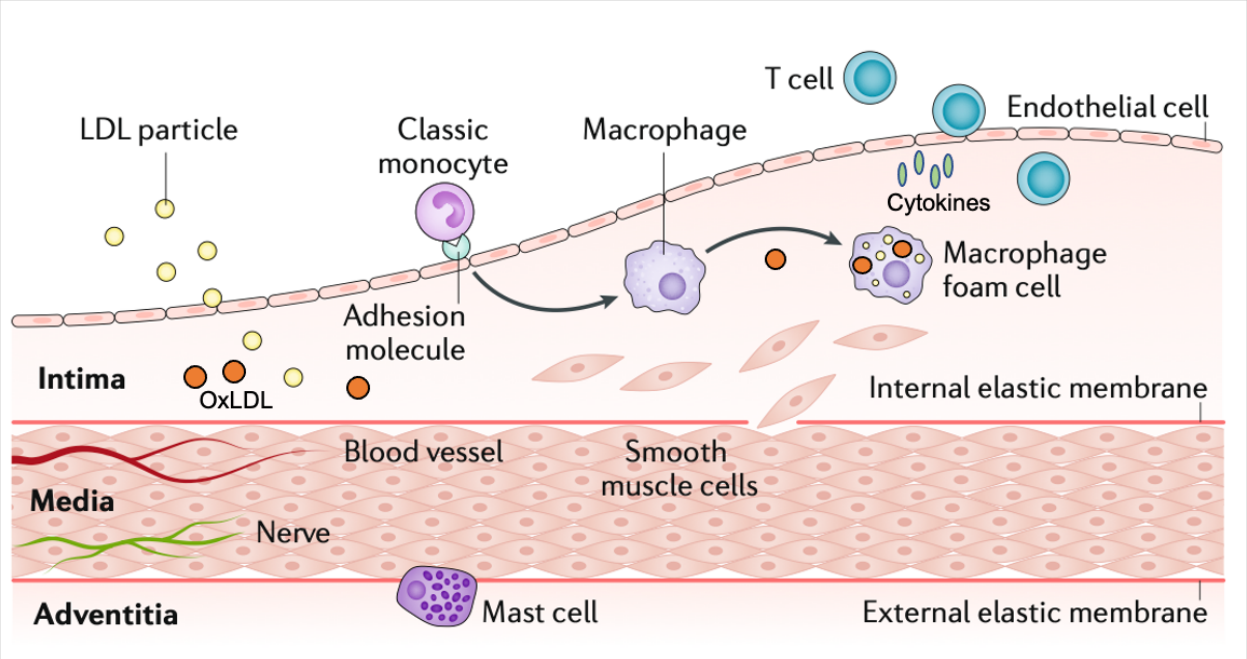


Figure 3[98]. Atherosclerosis physiopathology. Increased levels of lipids in plasma and low shear stress causes endothelial damage, allowing LDL particles to migrate through the subendothelial space, after getting oxidized (OxLDL), they induce the recruitment of monocytes that acquire macrophage characteristics and phagocyte the cholesterol particles becoming foam cells (FC). High levels of intracellular OxLDL and cholesterol promote macrophage apoptosis and a release of proinflammatory cytokines, leading to the migration and differentiation of vascular smooth muscle cells.

1.1.4.3 Cells involved in plaque progression

-T cells

T cells are critical drivers and modifiers of the pathogenesis of atherosclerosis. Naive CD4+ T helper (TH) cells are primed in secondary lymphoid organs. Antigen-presenting cells (APCs), such as macrophages and dendritic cells [133], take up and process oxidized LDL (oxLDL) in the plaque, migrate to the draining lymph node and present peptides from ApoB on major histocompatibility complex (MHC) class II molecules [134]. Naive T cells recognize this complex through their specific T cell receptors (TCRs). Co-stimulatory molecules induce T cells to express transcription factors that favour the differentiation phenotype of effector T (Teff) cells

or regulatory T (Treg). Consequently, T cell recruitment to the atherosclerotic plaque occurs via chemokines and chemokine receptors such as CCR5, CXCR3 and CXCR6 [135, 136].

A large body of evidence indicates that T helper 1 (TH1) cells have pro-atherogenic effects, express T-box transcription factor TBX21 (T-bet) and secrete interferon-γ (IFNγ), IL-2, IL-3, tumour necrosis factor (TNF) and lymphotoxin, which can all activate macrophages, T cells, and other plaque cells and thereby accelerate the inflammatory response [137-139]. Deficiency of IFNγ, or T-bet protects mice from atherosclerosis[135, 140, 141] and IFNγ could directly reduce plaque stability by inhibiting vascular smooth muscle cell (VSMC) proliferation[142] . Also, TH1 can differentiate into Treg cells with anti-atherogenic roles. This subset is characterized by expression of the transcription factor forkhead box protein P3 (FOXP3) in mice. Treg cells protect against atherosclerosis by secreting high levels of IL (interleukin)-10, TGF (transforming growth factor)-β. IL-10 is an anti-inflammatory cytokine, whose deficiency increases atherosclerosis in atheroprone mice [143-145]; TGFβ has plaque-stabilizing effects in mice by suppressing the proliferation of pro-inflammatory effector T cells [146, 147]. However, Treg cells can become pro-atherogenic by losing the expression of CD25 and FoxP3 induced due to high intracellular cholesterol levels or decreased IL-2 signaling [148, 149]. The roles in atherosclerosis of other TH cell subsets such as TH2, TH9, TH17, TH22, follicular helper T cells and CD28– T cells, as well as other T cell subsets including CD8+ T cells and γδT cells, are less well understood. Moreover, some T cells seem to have both pro- atherogenic and anti-atherogenic functions.

- Vascular smooth muscle cells

VSMCs are the main cell type in early arterial intimal thickenings and a major component of most stages of human atherosclerosis [150, 151]. The VSMCs are not terminally differentiated and can change their phenotype in response to environmental cues including growth factors/inhibitors mechanical influences, cell-cell and cell-matrix interactions, extracellular lipids and lipoproteins, as well as various inflammatory mediators in the injured arterial wall [152, 153].

Normally, VSMCs proliferate and migrate at an extremely low rate. Contractile VSMCs within the atherosclerotic plaques are mainly localised at the fibrous cap and express VSMC-

specific genes, which include α-, γ-SMA (smooth muscle actin), calponin, SM22-α, smoothelin, and SM-MHC (smooth muscle myosin heavy chain)[154]. Macrophages clearing dead cells produce proresolving mediators, such as IL-10, TGF-β, and specialized proresolving lipid mediators (SPMs). These, in turn, cause VSMCs to differentiate and deposit ECM (extracellular matrix), this plays a beneficial role in plaque stabilization by forming the protective ECM-dense fibrous cap comprise mainly by laminins, polymerized collagen I, collagen III, collagen IV, small amounts of fibronectin, and elastin fibers [155-157].

While positive remodeling during atherosclerosis progression preserves vessel patency by expanding outwards, negative remodeling, which occurs once the vessel has expanded by >40%, occludes lumen diameter as atherosclerosis advances [158]. When the plaque progresses, VSMCs migrate from the media toward the intima where they proliferate and acquire a synthetic phenotype. They gradually lose the α-SMA, marker of VSMC lineage [159-161] and start expressing macrophage markers, such as CD68. The transition toward a synthetic phenotype result in a gradual shifting from a differentiated to a dedifferentiated state acquiring the ability to uptake lipids and to become VSMC-derived foam cells [162-164]. They contribute >50% of total plaque foam cells [165]. Lineage-tracing experiments in advanced atherosclerotic lesions demonstrated that SMCs make up a much larger part of lesioned foam cells than previously thought [166-168].

1.1.4.4 Plaque apoptosis and necrotic core

As the lesion matures and advances it becomes known as an atheroma and contains many dead cells and debris forming the necrotic core all of which is contained within an extensive ECM acting as a trap for all these cells [169]. Also, macrophage foam cell apoptosis due to ER stress from the lipids [170]. Plaque VSMCs number is reduced due to apoptosis causing less collagen secretion [169]. The large accumulation of dead cells remains in the plaque and are not cleared due to defective efferocytosis which leads to the necrotic core. Large necrotic cores are characteristic of an advanced plaque can cover over 25% of the lesion area [171].
Eventually, the growing mass of lipids and apoptotic cells overwhelms the macrophage phagocytic capacity. These dying cells swell and burst open, releasing stores cell debris and lipids, along with

pro-inflammatory cells [172-179]. A summary schema of the physiopathology is presented in Fig. 3 [98].

Other common features of advanced plaques include calcification; surface fissures and ulcers, which can release lipids from deep within the plaque; hematomas and hemorrhages; and thrombotic deposits [180-183]. The most dangerous outcome for atherosclerotic plaques is for them to rupture and release their pro-inflammatory and pro-thrombotic contents into the circulation. This forms a thrombus that can cut off circulation either at the site of the rupture or in smaller downstream arteries, such as the coronary arteries supplying the myocardium or the cerebral microvasculature, resulting in tissue death [1, 183]. Vulnerable plaques tend to have a large necrotic core, high macrophage content, and a thin fibrous cap with reduced collagen and SMC content [181, 184-186]. Plaques are most fragile at the edges [187, 188] and particularly on the upstream side, a region of high shear stress. This suggests that while low shear stress plays a pivotal role in the initial lesion formation, high shear stress promotes plaque rupture [189-191]. Indeed, high shear stress upregulates matrix metalloproteinases (MMPs), which degrade the fibrous cap [192]. Interestingly, the pro-inflammatory milieu also stimulates the release of MMPs by macrophages [193].

1.2 Atherosclerosis and gut microbiota

1.2.1 Microbiota

The human body is colonized by a variety of microorganisms collectively referred as microbiota. Approximately 100 trillion cells and only 10 trillion are human cells while 90 trillion are microbes [194]. The indigenous microbiota is composed of archaea, bacteria, viruses, protozoa and fungi [195], which form a network with many interactions. The genes of these microorganisms form our metagenome, known as our second genome. The humans have approximately 3 million microbial genes (150 times more genes than the human genome) encoding various types of proteins that play a critical role in essential physiological processes, such as immune system maturation, food digestion, drug metabolism, detoxification, vitamin production, and prevention of pathogenic bacteria adhesion [196, 197]. Gut microbiota is a complex community of microorganisms that live in the digestive tract. In people, the gut microbiota has the biggest quantities of microorganisms, and the greatest number of species compared to other parts of the

body. The human gastrointestinal tract is known to host trillions of microbes , the number of which reaches approximately 10^{14} cells in the entire gut of a healthy individual [198]. The relation between the gut microbiota and human health is being increasingly recognised because studies have shown that a healthy gut flora is largely responsible for overall health of the host [199]. Colonization of the gastrointestinal tract begins after birth. It is known that colonization initiates from maternally acquired bacteria during birth [200] and breastfeeding and continues throughout our life [201-203]. Most of the bacteria in the gut are anaerobes (99%)[204].

Over the lifetime of the individual, or at least until stabilization of colonizing microbiota in adulthood, there is a change in the profile of the predominant phyla in the gastrointestinal tract, migrating from a community dominated by Actinobacteria and Proteobacteria to one dominated by Firmicutes and Bacteroidetes [205, 206]. Some studies have described that the gut microbiota of obese animals and humans exhibits a higher Firmicutes/Bacteroidetes ratio compared with normal-weight individuals [207, 208]. For example, children living in rural African areas, who consumed a traditional diet rich in fiber and showed higher proportions of Bacteroidetes and lower of Firmicutes, compared to children from western countries whose diet included large amounts of protein, fat, sugar, and starch [209] proposing this ratio as health biomarker [210].

1.2.2 Metabolism of gut microbiota

The microbiota is responsible for metabolizing dietary elements into bioactive food components. Gut bacteria could metabolize indigestible carbohydrates such as cellulose, hemicelluloses, resistant starch, pectin, oligosaccharides, and lignin into SCFAs composed of acetic, propionic and butyric acids [211, 212]. These metabolic products are mainly produced by Firmicutes, Bacteroidetes and some anaerobic gut microorganisms [213]. The microbiota also exhibit beneficial effects on the host organism, in terms of helping vitamin synthesis such as biotin, thiamine, cobalamin, riboflavin, nicotine and pantothenic acids, vitamin B and K [214]. Carbohydrates, branched chain amino acids, amines, phenols, indoles and phenylacetic acid as well as bile acids, cholesterol and conjugated fatty acids are also generated through the action of gut microbiota [215] [216]. Furthermore, it has been reported that gut microbiota has the capacity to synthesize some neurochemicals that can affect the central nervous and peripheral enteric systems, such as serotonin and GABA [217, 218].

1.2.3 Gut microbiota and diseases

Gut microbiota have been associated with a large array of human diseases such as inflammatory and irritable bowel diseases [206, 219, 220]. In the inflammatory bowel disease, a direct relationship between the diet and microorganisms has been demonstrated. Irritable bowel syndrome is caused by increased short chain fatty acids (SCFAs), that enhances the release of serotonin from the intestinal mucosa increasing intestinal transit [219, 220] and gut microbial dysbiosis [221]. In addition, gut microbiota has been involved in metabolic diseases such as obesity and diabetes [222], allergic diseases [223] and neurodevelopmental illnesses [224]. Interestingly, gut microbiota has been shown to influence atherosclerosis.

1.2.3.1 Bacterial infections and atherosclerosis

The presence of bacterial DNA such as C. pneumoniae, Firmicutes and Proteobacteria phyla in human atherosclerotic plaques has been demonstrated. However, whether an infection initiates or augments development of plaques is uncertain and the correlation between plaque morphology and bacterial content is still under investigation [225]. In animal models, microorganisms such as A. actino mycetemcomitans, C. pneumoniae, H. pylori, and P. gingivalis might contribute to atherosclerosis by increasing lesion areas [226]. In addition to infections and bacteria in atherosclerotic plaques, the gut microbiota also influences CVD through the regulation of host metabolism, including cholesterol and lipid metabolism. Indeed, gut microbiota is now thought to have a pivotal role in low-grade inflammatory metabolic diseases such as obesity and diabetes [227-229] which are associated with CVD [230].

1.2.3.2 Gut microbiota, inflammation and atherosclerosis

Studies in rodents and humans showed that chronic consumption of HFD leads to intestinal barrier defects that facilitate the passage of intestinal luminal contents (food antigen, bacterial products, bacteria), and bacterial lipopolysaccharide (LPS) into systemic circulation. LPS, also known as endotoxin, is structural compounds in the outer membrane of Gram-negative bacteria [228, 231]. LPS induces inflammation through activation of its receptor, TLR4, which is expressed on immune cells such as macrophages as well as on many other cell types including

hepatocytes and adipocytes. The intestinal epithelium works as a barrier to prevent translocation of bacterially derived factors. However, weight gain, HFD [232] and increased exposure of fatty acids [233, 234] may disrupt the gut barrier permeability allowing leakage of LPS [228]. This condition leads to metabolic endotoxemia [235], such as dyslipidemia, insulin resistance, non-alcoholic fatty liver disease and CVD [236]. Mice lacking the TLR4 co-receptor CD14 are resistant to hyperinsulinemia, insulin resistance and steatosis induced by a HFD or LPS [228]. The capacity of HDL to bind LPS may protect against inflammation. Indeed, infusion of HDL prior to a LPS challenge reduced release of proinflammatory cytokines in humans [237]. LPS has been reported to promote atherosclerosis by inducing pro inflammatory cytokines in different experimental models [238-240] . Stimulation with LPS leads to the recruitment of adaptor proteins such as myeloid differentiation primary response protein MYD88 to the cytoplasmic domain of TLRs. The recruitment of adaptor proteins triggers downstream signaling cascades that lead to production of proinflammatory cytokines and chemokines [241, 242].

1.2.3.3 Diet, microbiota metabolites and atherosclerosis

Western diet is characterized by high intakes of refined sugars (candies and sweets, and high-sugar soft drinks), animal fats (high intake of saturated and omega-6 fatty acids, reduced omega-3 fat intake), processed meats (red meat), refined grains, high-fat dairy products, conventionally-raised animal products, salt, eggs, potatoes, corn, mainly processed, refined, fried, and pre-packaged, with low intakes of unprocessed fruits, vegetables, whole grains, grass-fed animal products, fish, nuts, and seeds. Hence, it is low in fiber, vitamins, minerals, and other plant-derived molecules such as antioxidants [243-245].

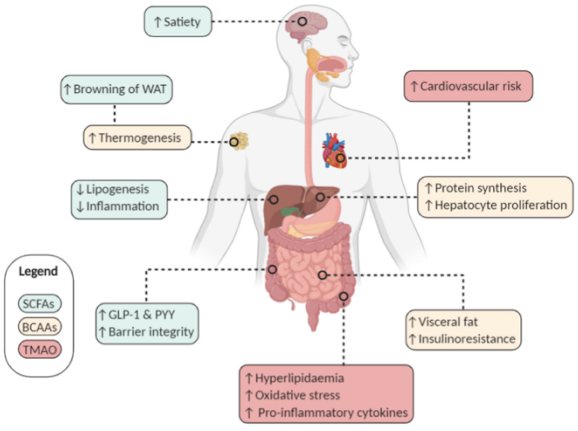


Figure 4. Gut microbiota metabolites.

Gut microbiota has been shown to differ between mice fed with a high or low fat diets and between diets containing equal amounts of fat but from different sources [235, 246, 247]. The influence of gut microbiota on host lipid metabolism may be mediated through metabolites produced by the gut microbiota such as SCFA, secondary bile acids and trimethylamine. Figure 4 [248] shows some systemic functions of gut microbiota metabolites.

- **SCFA**

SCFAs such as acetate, propionate and butyrate are bacterial metabolites derived from fermentation of fibers in the colon. SCFAs are important for host metabolism and are used as substrates for energy production, lipogenesis, gluconeogenesis and cholesterol synthesis [249, 250]. Butyrate is an energy source for colonocytes while propionate is mainly metabolized by the liver [251, 252].

In addition to being metabolic substrates, SCFAs have been shown to have a positive impact on metabolic health [253]. They act as signaling molecules, notably through the G-protein coupled receptors GPR43/FFAR2 that protect against diet-induced obesity in mice and GPR41/FFAR3. GPR43 [254-257]. Activation of GPR43 on L-cells , which are located along the length of the intestinal epithelium, make contact with the gut lumen via apical processes and are believed to respond directly to luminal signals [258], increases secretion of glucagon-like peptide-1 (GLP-1) [254, 259] and acetate induces anti-lipolytic activity [260] and improves glucose and lipid metabolism [255] through GPR43 in white adipose tissue. GRP41 has also been shown to regulate metabolism through interaction with the gut microbiota. Conventionally raised Gpr41 knockout mice are leaner and weigh less than their wild-type littermates, while these differences are not found in germ free mice. Furthermore, the microbiota increases peptide YY (PYY) production through GPR41 [261]. Supplementation with acetate re- duces weight gain and improves glucose tolerance in obese and diabetic rats [262], butyrate protects against obesity and increases thermogenesis in mice [263] and propionate or butyrate improves glucose homeostasis in mice [264].

- Bile acids

Bile acids are others gut microbiota-derived metabolites involved in various metabolic diseases [265]. Primary bile acids are synthesized from cholesterol and conjugated to taurine or glycine in the liver. They are stored in gallbladder and excreted into the duodenum after food ingestion to aid emulsification of dietary lipids. Most bile acids are reabsorbed and recirculated to

the liver, but Intestinal anaerobes mediate a deconjugation of the glycine or taurine group catalyzed by bile salt hydrolases (BSH) reduces reabsorption [266, 267]. Deconjugated bile acids can be further metabolized to secondary bile acids through dehydrogenation, dihydroxylation and epimerization by colonic bacteria [266]. These secondary bile acids are highly insoluble and toxic and are mostly excreted in the feces and As a result, to replace the amount lost, roughly 0.5 grams per day is synthesized from cholesterol in the liver [267]. Suppression of hepatic bile acid biosynthesis may also inhibit HFD-induced gut microbiome alterations. Feeding mice with bile acids under a normal diet induced an obese phenotype, showing the presence of the liver–bile acid–gut microbiome metabolic axis [268]. Recently, the bidirectional relationship between gut microbiota and bile acid metabolism [269] in CVD has been reviewed [270]. Bile acids can accelerate atherosclerosis development through bile-salt hydrolase activity and bile acid receptors [271, 272]. Bacteria-mediated bile salt hydrolase activity can promote atherosclerotic progression by stimulating cholesterol accumulation, foam cell formation, and increasing the atherosclerotic plaque size [273-276].

The microbiota-derived secondary bile acids play essential roles in atherosclerosis development by modulating various bile acid receptors such as farnesoid X receptor (FXR), pregnane X receptor (PXR), and vitamin D receptor (VDR), as well as membrane receptors Takeda G protein receptor 5 (TGR5) and sphingosine-1-phosphate receptor 2 (S1PR2) [277].–TGR5 expressed in macrophages is an important bile acid receptor of the host that mediates the systemic effects of bile acids [278]. Its activation can inhibit atherosclerosis development by reducing macrophage-mediated inflammation and lipid loading [279].

- Trimethylamine

Gut microbiota metabolizes methylamine-containing nutrients such as choline, lecithin and L-carnitine, contained in many foods, mainly in red meats, such as beef and lamb [280], to generate trimethylamine (TMA), which is further processed to trimethylamine N-oxide (TMAO) by flavin monooxygenases (FMO) in the liver [281] (Figure 5 [282]). TMAO levels have been correlated with atherosclerosis, with risk of cardiovascular events, and with prevalence of cardiovascular disease [283, 284]. Plasma TMAO levels in different mouse strains have been positively correlated

with lesion size [285] and transfer of microbiota from high and low TMAO producing mice to atherosclerosis prone ApoE-/- mice showed increased microbial capacity for TMA production and aortic lesions [286]. FMO3 is the primary enzyme converting TMA into TMAO. Knockdown of FMO3 results in reduced atherosclerotic lesion areas, altered lipid and cholesterol metabolism, and decreased TMAO plasma levels [287, 288]. FMO3 expression is regulated by bile acids by a mechanism that involves FXR mentioned before [289]. Gut microbiota processing of bile acids could therefore be an alternative mechanism by which the gut microbiota regulates TMAO production. The disturbance of the biosynthesis of short chain fatty acids causes many pathological consequences for the host [290].

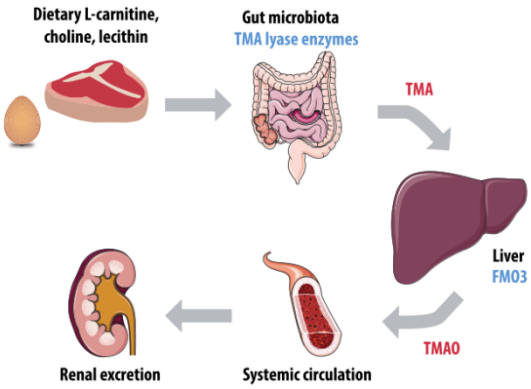


Figure 5. Production of trimethylamine-N-oxide (TMAO).

The mechanisms by which TMAO contributes to atherosclerosis appears to be complex and not fully understood. Antibiotic treatment reduces production of TMA and has been shown to suppress foam cell formation [281]. TMAO can also contribute to atherosclerosis by inhibiting reverse cholesterol transport increasing cell surface expression of two proatherogenic scavenger receptors, cluster of differentiation 36 (CD36) and scavenger receptor A (SR-A) [283, 291-293] and by inducing atherosclerosis-promoting inflammatory protein kinase, extracellular signal-related kinase, and nuclear factor-κB signaling cascade in vascular cells [294].

1.3 D-mannose

1.3.1 Diet sugars and physiopathology

Sugars are a common component of foods and beverages. Sugar- sweetened foods such as cakes, cookies, chocolate, ice creams, and drinks including soft drinks, energy drinks, fruit punches, lemonade, and iced tea are very popular in Western society [295]. A rise in the consumption of refined sugars in food and beverages has often been implicated in the epidemic of obesity, type 2 diabetes and CVDs. Monosaccharides are constituents of such widely abundant biomolecules as starch, cellulose, pectin and chitin. They are units of glycoprotein sugar chains

and other important glycoconjugates in bacteria and eukaryotic cells, and are also key components of DNA and RNA [296].

While most added sugars are consumed as sucrose (disaccharide) or glucose and fructose (monosaccharide constituents), it is the fructose fraction that is considered the more harmful sugar component, despite its classification as a low-glycemic sugar, [297]. A high glucose index has been associated with insulin resistance, cardiac remodeling or dysfunction, endothelial dysfunction of large and small arteries and atherosclerosis [298, 299] . Fructose supplementation to the diet of non-human primates for up to 1 year produced many features of the metabolic syndrome, including increased body weight, fat mass, insulin resistance, dyslipidemia with hypertriglyceridemia and decreased HDL cholesterol, and diabetes [300, 301]. A moderate increase in fructose consumption (75 g or 300 kcal per day) in men with obesity for 12 weeks increased body weight, liver fat, hepatic de novo lipogenesis, atherogenic dyslipidemia, and indices of insulin resistance [302-304]. D-mannose (hereafter referred to as mannose) is a natural C-2 epimer of glucose, is a monosaccharide approximately a hundred times less abundant than glucose in human blood [305]. Free mannose is found in small amounts in many fruits such as oranges, apples and peaches [306]. Physiological mannose concentration in human blood is ~50 μM, and it does not contribute significantly to cell bioenergetics [307].

Mannose supplementation, at safe supraphysiological doses up to 0.2 grams mannose/kg body weight, was shown to help in some human diseases such as carbohydrate-deficient glycoprotein syndrome type I [308]. It is also therapeutically effective as a non-antibiotic treatment for recurrent urinary tract infections in humans, by preventing adhesion of enteric bacteria to uroepithelial cells. Mannose can affect bacteria by combining with FimH protein, which is the type 1 pilus adhesin of Escherichia coli [309-311]. Dietary mannose is used to treat glycosylation deficient patients with mutations in phosphomannose isomerase [312]. In addition, mannose is used for N- and O-glycosylation, C-mannosylation and GPI anchor synthesis [307].

1.3.2 Metabolism of mannose

Mannose is transported into mammalian cells by diffusion via hexose transporters of the SLC2A group (GLUT) present primarily on the plasma membrane. No mannose-specific or -preferential transporters have been found among the 14 distinct GLUT transporters in humans [313]. Within the cell, mannose is phosphorylated by hexokinase (HK) to produce mannose-6-phosphate (Man-6-P), which serves as a common substrate for three competing enzymes. Man-6-P is either catabolized by phosphomannose isomerase (MPI) 95–98% [314, 315] or directed into N-glycosylation via phosphomannomutase (PMM2). Another minor pathway utilizes mannose for synthesis of 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (KDN), a sialic-acid related molecule found in fish and mammals [316] (Figure 6 [307]). The fate of Man-6-P largely depends on the ratio of MPI to PMM2 within a cell [315] - higher ratio leads to greater catabolism, while lower ratio favors the glycosylation pathway. However, the regulation of this ratio does not seem to be cell specific, but in some patients with PMM2-congenital disorder of glycosylation, an alteration of some immune cell populations, such as an increase of neutrophils, and natural killer cells has been described [317, 318]. PMM2-derived mannose-1-phosphate (Man-1-P) is then incorporated into several glycosylation intermediates including GDP-mannose (GDP-Man), GDP-fucose, and dolichol phosphate mannose (Dol-P-Man). These intermediates then contribute to N-glycosylation, O- glycosylation, C-mannosylation, and GPI anchor synthesis [307].

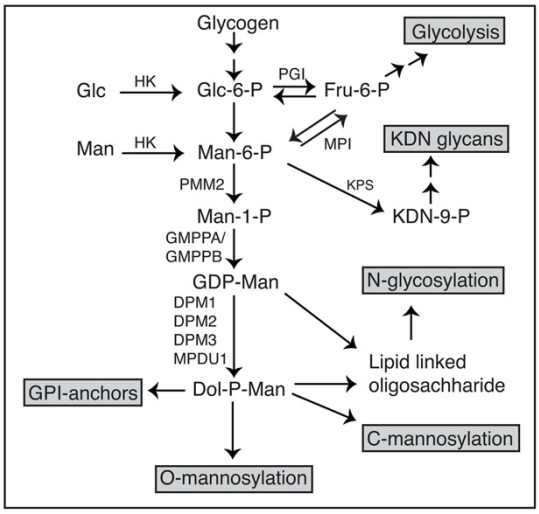


Figure 6. Mannose metabolism.

1.3.3 Mannose and inflammation

Mannose exhibits strong anti-inflammatory properties in different studies. Oral administration of mannose in mice is effective in decreasing *in vivo* growth of multiple tumor types and in potentiating the effects of cytotoxic chemotherapies, by accumulation of mannose-6-phosphate within cells. Furthermore, *in vitro* studies have shown that mannose induces tumor cell

death by apoptosis, through disruption of Bax and Bak mitochondrial proteins in combination with some chemotherapeutic drugs [319, 320].

The anti-inflammatory properties of mannose are also through regulation of immune cells. Mannose was demonstrated to affect T cell function by promoting generation of regulatory Foxp3+ T cells from naive CD4+ T lymphocytes and downregulates effector T cell activation [321, 322]. It also suppresses T cell-mediated immunopathologies in mice such as osteroporosis, lupus, autoimmune diabetes and airway inflammation, by promoting activation of the latent form of TGF-β [323, 324].

Importantly, mannose reduces LPS-induced macrophage activation by impairing IL-1ß gene expression[305]. Mannose has been shown to decrease the number and activation of proinflammatory myeloid cells infiltrating the colon in a dextran sulfate sodium-mouse model, by impairing glucose metabolism and raising intracellular mannose-6-phosphate levels. [325-328]
In addition, mannose inhibits wound inflammation by reducing the number of neutrophils and by inhibiting the synthesis of hyaluronic acid [329] .

1.3.4 Mannose and gut microbiota

A relationship between mannose and gut microbiota composition was recently reported. Indeed mannose is able to reconstruct the gut microbiota and to change metabolite composition [321]. Freeze and his team were studying mannose in the context of a rare disease called a congenital disorder of glycosylation (CDG). People with a specific form of the disease can be treated with mannose. While conducting their research, the scientists observed the anti-obesity effects of mannose feeding [330].

A closer look by Sharma and coauthors revealed that C57BL/6 mice were also protected from typical negative effects of a fatty diet. They had less body fat, reduced fat in their liver, stable blood sugar and even improved fitness. Surprisingly, these benefits were only seen when the mice received mannose early in life; older mice didn't benefit from mannose [331-333]. The protective effect of mannose on HFD-induced obesity is explained by an increase of Bacteroidetes and Firmicutes ratio in the gut. In the same line other studies reported the same changes in this ratio

in the intestines of obese mice or humans [331-333]. Another explanation could be that mannose also converts complex glycans to monosaccharides, inhibits glycosyl hydrolases leading to a higher fecal energy (fuel value expressed in KJ/gram) [334].

2. Hypothesis

Atherosclerosis is a chronic inflammatory disease influenced by gut microbiota. D-mannose has anti-inflammatory properties and induces changes in gut microbiota preventing the deleterious effects of high cholesterol diet.

We hypothesize that oral D-mannose supplementation to high fat diet protects against atherosclerosis development through regulation of gut microbiota composition and inflammation.

Research aims

- Aim 1: Determine the effects of mannose supplementation on atherosclerotic plaque size and composition

- Aim 2: Determine the effects of mannose supplementation on gut flora diversity.

- Aim 3: Determine the effects of mannose supplementation in plasma, peritoneal cavity and bone marrow cells.

3. Methods

3.1 Mouse handling, high fat regimen and mannose supplementation.

ApoE-/- mice of the C57BL/6 strain were obtained from The Jackson Laboratory and bred in the animal facility at the Lady Davis Institute. All personnel involved in handling animals and performing experiments have completed the necessary training modules required by the McGill University Animal Care Committee. Furthermore, all experiments and methods of handling conform to the guidelines set forth by the Animal Care Committee as well as to the protocol of the present research project. Eight weeks old male and female mice were given a HFD (15% cocoa butter fat, 0.5% cholesterol). Baseline body weight was measured and mice were divided in two groups according to the route of mannose administration.

1. 0 (Ctl), 5, or 20% of mannose was supplemented orally in drinking water for 4 or 9 weeks (n=18-28).

2. 5g/kg mannose/PBS were injected intraperitoneally (IP) for 9 weeks-5 times a week (n=8-9).

Body weights, drink and food intake were monitored throughout the experimental period. After 9 weeks of treatment mice were anesthetized by isoflurane and then euthanized by CO^2 asphyxiation followed by cervical dislocation. Body weight was recorded, and cardiac puncture was performed to collect 0.5ml of whole blood in EDTA-coated tubes (Sarstedt, Numbrecht, Germany) and centrifuged at 2,000 rpm at 4 °C for 20 min to collect the plasma. PerC cells were obtained (see below). To obtain peripheric monocytes, lymphocytes and granulocytes, blood cell enumeration was performed with a hematology analyzer (scil Vet abc).

The peritoneal cavity was opened, and small intestine (duodenum, jejunum and ileum) was collected. Next, the thoracic cage was opened, and the heart was flushed with PBS to rinse the vasculature. Common sites for atherosclerotic plaques such as, the aortic sinus, the brachiocephalic artery (BCA) and the ascending aorta, were dissected [335] (Fig. 7 [336, 337])

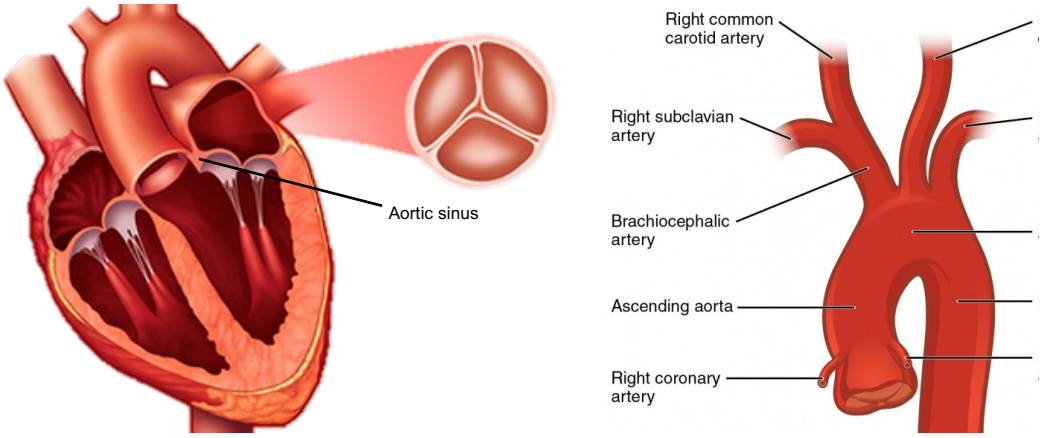


Figure 7. Aortic sinus [336] and brachiocephalic artery [337] anatomic locations for collection.

After removing other overlying tissues from the exposed thoracic cavity (e.g. lungs, thymus, adipose tissue, vena cava), the BCA and the aortic sinus were collected to evaluate atherosclerotic plaque size (Oil red O staining) and composition in macrophages, smooth muscle cells (immunocytochemistry) and collagen (Pico Sirius Red and Masson's trichrome staining).

Aortic sinus, BCA and pieces of small intestine were fixed in 4% paraformaldehyde for 24 hours before being placed into 30% sucrose solution at 4 °C for 24 hours to prevent the formation of structurally disruptive water crystals. tissues were then embedded in OCT gel.

3.2 Plasma lipids, mannose and LPS

To measure plasma lipids: Cholesterol Assay Kit ab65390 was used, which is a quantitative colorimetric/fluorometric method to quantify total cholesterol, free cholesterol, and cholesterol esters in mammalian samples. To measure plasma mannose: Mouse mannose (MN) ELISA Kit was used, which is an in-vitro enzyme-linked immunosorbent assay for the quantitative measurement of mouse mannose receptor.

To measure LPS levels: Pierce™ Chromogenic Endotoxin Quant Kit was used measured. It is an end-point chromogenic endotoxin detection assay based on the amebocyte lysate method, which measures endotoxin through the interaction of the endotoxin with the proenzyme Factor C found in circulating amebocytes of the horseshoe crab.

3.3 Gut microbiota composition

To analyze gut microbiota composition, mice stools were collected at days 0 and 60 of treatments. Mice were placed in previously sterilised glass beakers, the stools were collected immediately and stored at -80°C. DNA was extracted and 16S ribosomal RNA gene sequencing was performed by McGill genome Centre. This technique utilizes PCR to target and amplify portions of the hypervariable regions (V1-V9) of the bacterial 16S rRNA gene. PCR amplicons from up to hundreds of samples are then combined and sequenced on a single run. The raw data showed the quantity of bacteria found in each sample which was organized according to the 5 most popular phyla. Data and graph were worked in excel.

3.4 Cryosectioning and tissue staining

OCT-embedded aortic sinus, BCA and intestine samples were serially sectioned into 7 µm slices at -23°C at tissue depths corresponding to the consistent location of plaques. Several microscope slides were collected from each sample to stain for different markers. Slides were stored at -80°C until the day of staining and were allowed to thaw at room temperature for 30 min before proceeding. To appreciate the intestinal macrophages jejunum slides were blocked with 5% bovine serum albumin in PBS for 30 min followed by immunofluorescence staining with F4/80 which is a general marker for macrophages [128] and DAPI (4′,6-diamidino-2-phenylindole) which is a blue-fluorescent DNA stain to visualize cell nuclei by fluorescence microscopy.

To quantify plaque size, aortic sinus and BCA sections were stained for 45 min in OilRedO to colorize lipids. Representative light microscope pictures were taken and analyzed by ImageJ, using the polygon selection tool to contour the plaques. Plaque areas were measured in mm^2 using Image J. For each sample, several sections plaque areas were averaged.

To quantify plaque contents, slides were blocked with 5% BSA for 30 min followed by immunofluorescence staining with antibodies targeting α-SMA for the expression of alpha smooth muscle actin cells [338] and CD68 [339] for macrophages and other mononuclear phagocytes. Images were taken with a fluorescent microscope. For appreciating the necrotic cores, sinuses were dyed with Masson's Trichrome stain which is a method used for the detection of collagen fibers and in atherosclerosis we used it to appreciate the necrotic cores (NC)[340]. In ImageJ, threshold

analysis was performed on the contoured plaque areas to obtain a percentage of signal positive area. Once again, these were averaged into a single value per sample. To obtain the plaque stability index α-SMA average value was divided per the NC average value, as seen in thew next formula: Plaque stability index= %α-SMA/%NC.

3.5 Extraction of primary bone marrow cells

Six- to eight-weeks-old C57BL/6 mice were anesthetized by isoflurane and then euthanized by CO2 asphyxiation followed by cervical dislocation. Both legs were detached from the pelvis and stripped of their muscle. Under a cell culture hood, the femurs and tibias were cut at both ends and flushed with DMEM + 10% FBS + 1% P/S (using syringes) to collect the bone marrow (BM) cells. They were centrifuged at 1,500 rpm for 5 min. The supernatant was aspirated, and the remaining cell pellet was resuspended in FACS buffer (2% FBS) ready for flow cytometry or frozen.

3.6 Peritoneal macrophage cells extraction

To observe peritoneal cell recruitment, we realized two variations of the treatment length. The first mice on HFD received mannose 0 (Ctr), 5 or 20% for nine weeks and euthanized. The second group on HFD and mannose 0 (Ctr), or 20% for 4 weeks, received a 1.5 ml peritoneal prewarmed injection of aged 4% thioglycolate. Four days later, mice were euthanized, and macrophages were collected by exposing the peritoneal cavity and injecting and re-aspirating 10 ml of 2% FBS in PBS, taking care not to puncture organs to avoid erythrocyte contamination. Cells were centrifuged at 1,500 rpm for 5 min, re-suspended, and counted before proceeding with experiments.

3.7 Western blot

Tight junctions TLR-4, claudin-1 and occludin were measured by western blot. Protein was extracted using a commercial kit. All samples were dosed on the day of the Western blot. Depending on the expected band sizes, samples were run on 8, 10, or 15% polyacrylamide gels and then transferred to a nitrocellulose membrane. After rinsing in tris-buffered saline with Tween 20 (TBST), membranes were blocked in 5% skim milk in TBST, followed by the addition of primary antibody in milk. Membranes were again washed in TBST and incubated with secondary

antibody in milk. After washing, membranes were covered with an enhanced chemiluminescence solution for 5 min in the dark, inserted into a plastic sleeve, and exposed with a chemiluminescent imaging machine. If required, blotted antibodies were removed with a stripping agent and the membrane was re-blocked and re-blotted with a new primary antibody. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or b-actin was blotted as a loading control. All rinsing, blocking, and antibody steps were performed on a rocking platform.

3.8 Flow cytometry

For flow cytometry, 1×10^6 cells were stained with a live/dead marker, blocked with Fc receptor, and stained with antibodies targeting surface markers according to the 4 different panels, see complete antibody information in Fig 8.

- Blood panel: CD11b, CD115, Ly6G, Ly6C, CCR2, CX3CR1, CD206.
- PeriC panel: CD45, CD38, F4/80, CD115, CD206, Ly6G, CD68.
- Bone Marrow panel: CD11b, CD34, Ly6C, Ly6G, Sca1, c-Kit, CD16/32, CD3, CD19, B220, CD161/NK1.1 and Tor119.
- Lamina propia: TLR4, CX3CR1, CCR2, CD64, F4/80, CD206, CD11c, Ly6C, Ly6G, CD45, MHCII.

Fluorescence minus one control was used to determine fluorescence background and positivity. Data analysis was performed using FlowJo software. Gating was first performed on forward versus side scatter to remove cell debris and doublets before selection of live cells based on exclusion of a viability dye, 7-aminoactinomycin D (BioLegend), or Aqua (BD Biosciences).

In blood, cells were selected based on their CD11b marker expression and Ly6G to differentiate from granulocytes. Different subtypes were detected based on their differential expression of markers. Ly6C+CD115± for monocytes (Supplemental fig.A). In the bone marrow cells were selected from Ly6C+ as described above to detect monocytes. After from live CD3-CD19-B220-CD161/NK1.1-Ter119- cells were selected. Among the rest of the cells c-Kit+Sca1- was selected for gating progenitor cells (Supplemental fig.B). In the PerC cells were selected based on their CD45+ and Ly6G- to differentiate from granulocytes. Among cells, F4/80 was used for macrophages (Supplemental fig.C). Samples were centrifuged at 1,500 rpm at 4 °C for 5 min

between each step (except between blocking and antibody steps), and all steps up until intracellular blocking and antibody were done on ice (the latter portion was done at room temperature). Proportions of immune cell subtypes such as monocytes, macrophages, granulocytes and T cells were measured in the blood, the peritoneal cavity and the bone marrow by flow cytometry.

3.9 Statistical methods

In vivo data are presented as mean ± standard error of mean. Given the variability between experimental runs for *in vitro* experiments, these values are presented as a ratio of the experimental condition over the control or vehicle condition to allow pooling of results across different samples and experiment days. Within each experiment, outlier testing was done using the ROUT method (in Prism 6 software). An unpaired, two-tailed T-test was used for experiments with exactly two conditions, whereas a one-way analysis of variance (ANOVA) was used for experiments with more than two conditions. In both cases, the threshold of statistical significance was chosen to be $p < 0.05$. For statistically significantly results by one-way ANOVA, Tukey's honestly significant difference post-hoc test was used to identify which pair(s) of conditions were significantly different, again at a threshold of $p < 0.05$.

Manufacturer	Host + conjugate	Biological target	Concentrations (Stock)	Clone
Invitrogen 48-0112-82	Rat eFluorTM 450 (Pacific blue)	Mouse, Human CD11b	0.2 mg/mL	M1/70
BioLegend (135531)	Rat APC/Cy7 conjugated	Mouse CD115	0.2 mg/ml	AFS98
Invitrogen 17-5932-80	Rat APC	Mouse Ly6C	0.2 mg/mL	HK1.4
BD Pharmigen 561105	Rat FITC	Mouse Ly6G	0.5mg/ml	1A8
Biolegend 150659	Rat PE	MouseCCR2	0.2mg/ml	SA203G11
Biolegend 149035	Mouse Alexa Fluor 700	Mouse CX3CR1	0.5mg/ml	SA011F11
Biolegend 141711	Rat Alexa Fluor 647 (PE)	CD206	0.5mg/ml	C068C2
Invitrogen 56-0451-82	Rat Alexa Fluor 700	CD45	0.2 mg/mL	30-F11
Biolegend 102732	Rat Brilliant Violet 421	CD38	0.2 mg/ml	90
Invitrogen 12-4801-82	Rat PE	F4/80	0.2 mg/ml	BM8
Biolegend 137008	Rat APC	CD68	0.2 mg/ml	FA-11
Biolegend 101229	Rat PerCP/Cyanide5.5	CD11b	0.2 mg/ml	M1/70
R&D systems FAB6518N-100UG	Rat Monoclonal	CD34	0.2 mg/ml	700011
Biolegend 108127	Rat Brillian Violet 421	Sca1	50ug/ml	D7
Biolegend 105847	Rat Brillian Violet 605	c-Kit	0.2 mg/ml	2B8
Biolegend 101327	Rat APC Cyanine7	CD16/32	0.2 mg/ml	93
Biolegend	Rat	CD3	0.2 mg/ml	17A2

100205	PE			
eBioscience 12-0193-82	Rat PE	CD19	0.2mg/ml	eBio1D3
Biolegend 103207	Rat PE	CD45R/B220	0.2 mg/ml	RA3-6B2
Biolegend 108707	Mouse PE	NK-1.1	0.2 mg/ml	PK136
Biolegend 116207	Rat PE	TER-119	0.2 mg/ml	TER-119
BD Bioscience 740812	Rat BV711	TLR4	0.2 mg/ml	MTS510
Biolegends 149023	Mouse Brilliant violet 421	CX3CR1	0.2 mg/ml	SA011F11
BD Bioscience 740622	Mouse BV650	CD64	0.2 mg/ml	X54-5/7.1
BD Bioscience 749283	Rat BUV737	F4/80	0.2 mg/ml	T45-2342
Biolegend 117333	Hamster BV605	CD11C	50um/ml	N418
Biolegend 128017	Rat PE Cyanine7	Ly6C	0.2 mg/ml	HK1.4
BD Bioscience 564279	Rat BUV395	CD45	0.2 mg/ml	30-F11
BD Bioscience	Rat BB700	MHCII	0.2 mg/ml	M5/114.15.2

Figure 8. Table of antibodies used for flow cytometry.

4. Results

4.1 Oral mannose supplementation did not change HFD-induced body weight increase and plasma lipids and increased plasma mannose levels.

ApoE$^{-/-}$ mice received a HFD supplemented orally with mannose 0% (Ctr), 5% and 20% for 9 weeks. No difference was observed in body weight at the end of the treatment, plasma LDL and total cholesterol (Fig.9A-C). Plasma mannose increased in mice supplemented with 5%. Surprisingly, we found no significant increase in 20% mice compared with Ctr (Fig.9D). HFD and drink intake were measured at mid treatment for 1 week. The 20% mannose-treated mice drink intake (ml/week/mouse) was diminished compared with Ctr (Fig.9E). However, HFD food intake was equivalent between groups (Fig.9F).

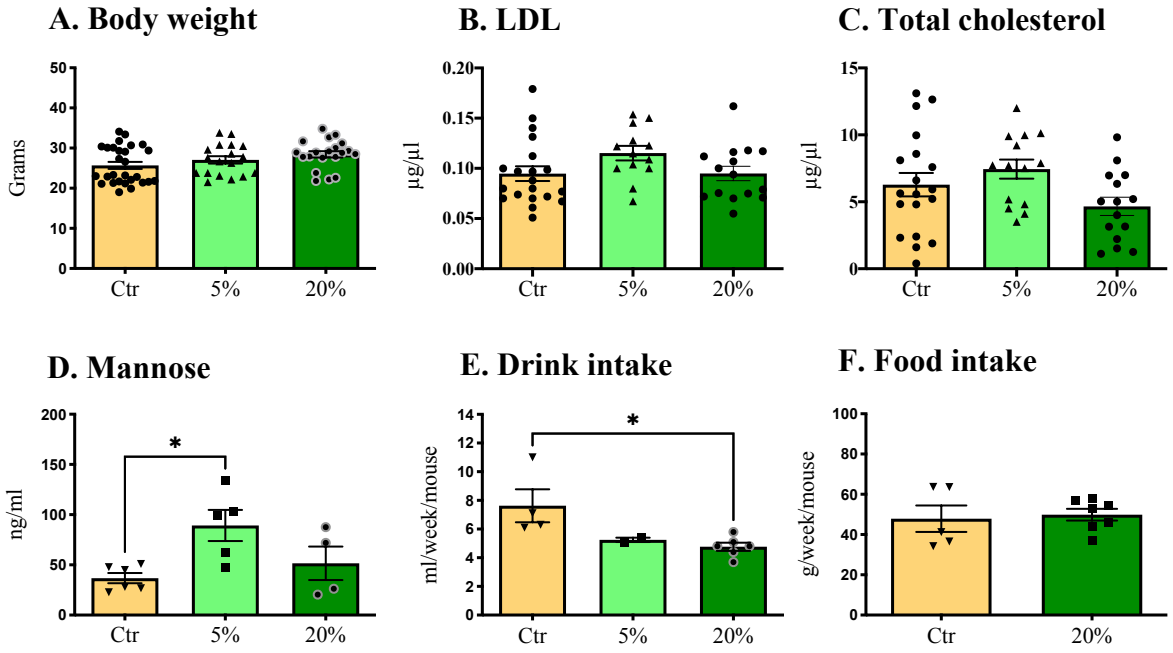


Figure 9. Animal parameters of ApoE$^{-/-}$ mice treated with HFD supplemented orally with 0 (Ctr), 5 or 20% mannose for 9 weeks. (A) Body weight (grams). (B) Plasma LDL levels (µg/µl). (C) Plasma total cholesterol levels (µg/µl). (D) Plasma mannose levels (ng/ml). (E) Drink intake (ml/week/mouse). (F) Food intake HFD food intake (gram/week/mouse). *P<0.05, n=2-28. Data are mean ± SEM (standard error of the mean).

4.2 Oral mannose supplementation prevented HFD-induced increase of F/B ratio.

First, we investigated whether mannose regulated gut microbiota. ApoE$^{-/-}$ mice received a chow or a HFD orally supplemented with mannose 20% for 9 weeks. 16S DNA sequencing on feces showed as expected two main populations of bacteria the Firmicutes and Bacteriodetes, and small amounts of Actinobacteriota and Verrucomicrobiota (Fig.10A). The Firmicutes/Bacteriodetes ratio was significantly increased in mice fed a HFD (HFD Ctr) (4.8±0.9) compared with Chow diet (Chow Ctr) (1.7±0.3). This increase was prevented in mice fed a HFD supplemented with 20% mannose (HFD + 20% mannose)(2.8±0.4) (Fig.10B).

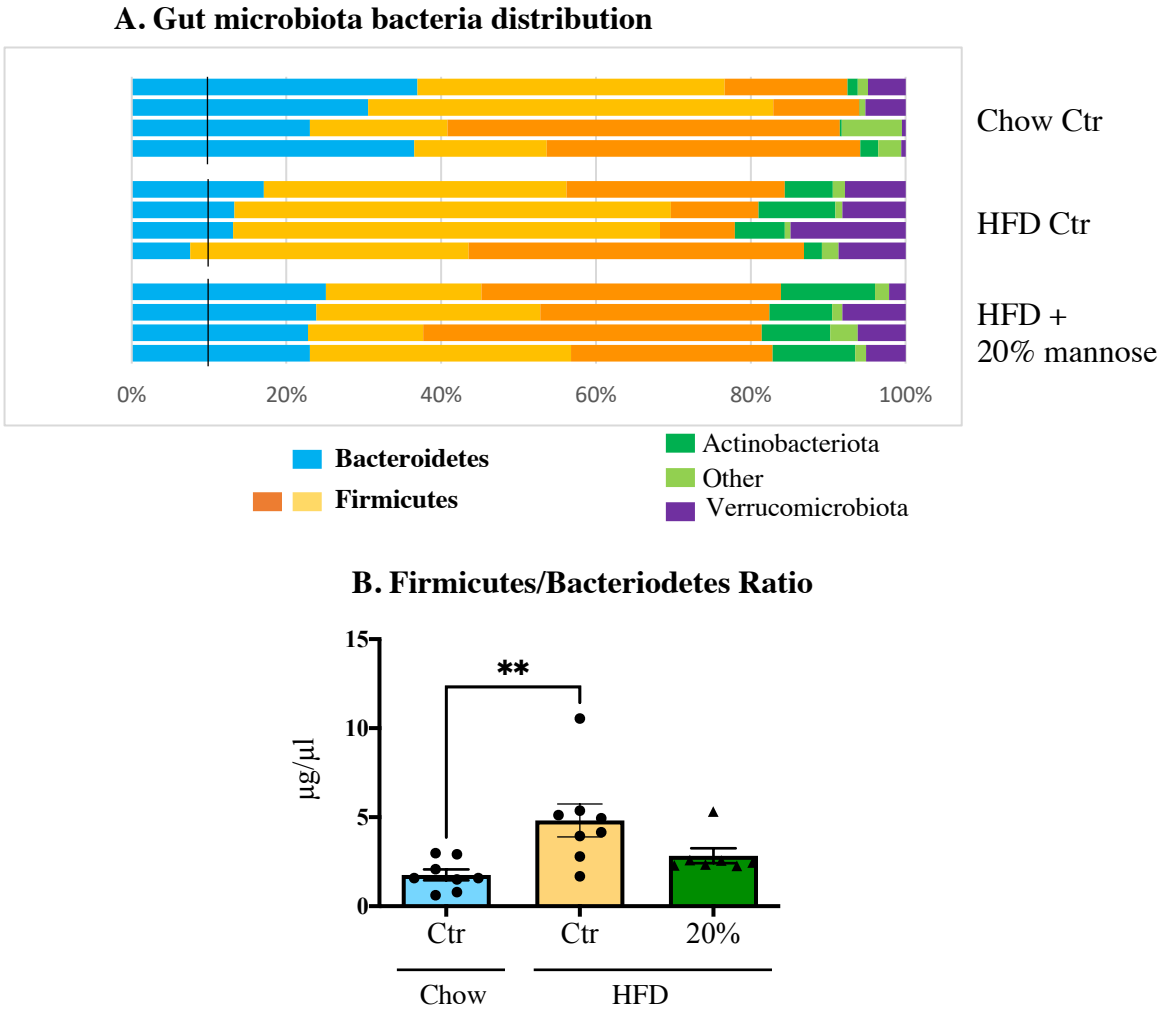


Figure 10. Fecal DNA P16S sequencing of ApoE -/- mice upon 9 weeks of chow diet (Chow Ctr), HFD (HFD Ctr) or HFD+mannose 20%. (A) Graph representing the distribution of different gut

microbiota bacteria. (B) Graph representing Firmicutes/Bacteroidetes ratio. **P<0.01, n=7-8. Data are mean ± SEM.

4.3 Oral mannose supplementation reduced proinflammatory macrophages in the small intestine and prevented plasma LPS increase.

To determine if the effects of mannose on gut microbiota composition was related to gut inflammatory state, we first assessed gut macrophages. Our preliminary data indicated a decreased number of F4/80 expressing macrophages in the intestine (Fig.11A). Interestingly, flow cytometry indicated a significant decrease of TLR-4 in the lamina propia of the small intestine, the receptor of LPS on F4/80+ macrophages in 5 (1.7±0.2) and 20% (2.1±0.4) supplemented mannose mice as compared with Ctr (4.9±0.7) (Fig.11B). In line with this results, plasma LPS levels increased 2-fold in HFD-treated mice (12±2.1) compared with chow diet controls (6±0.4) and 20% mannose (8.7±1.5) diminished this increase (Fig.11C).

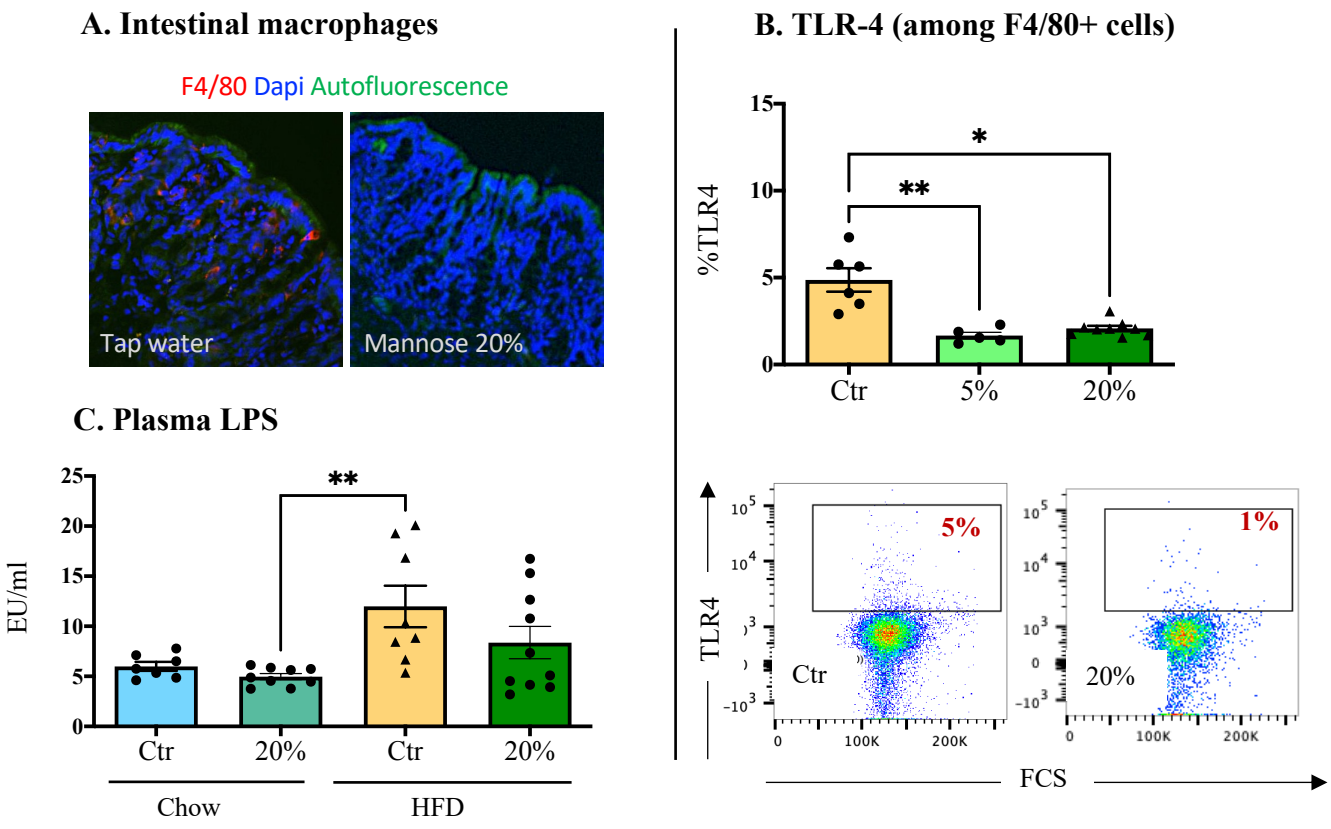


Figure 11. ApoE$^{-/-}$ mice were fed treated a chow diet (Ctr Chow) or HFD supplemented orally with 0 (Ctr), 5 or 20% mannose for 9 weeks. Plasma and intestines were collected. (A) Immunofluorescent staining of intestinal sections with F4/80+ macrophages (in red) and Dapi for nuclei (in blue). (B) Flow cytometry representative dot plots and quantifications of TLR4 on F4/80+ macrophages in the lamina propia of the small intestine. (C) Plasma lipopolysaccharide (LPS) levels by end point assay (endotoxin units-EU/ml). *P<0.05, **P<0.01 n=5-10. Data are mean ± SEM.

4.4 Oral mannose supplementation does not have an effect in the intestine permeability.

No difference in TLR-4 expression in the intestine as well as the tight junction proteins, claudin-1 and occludin was observed between mannose-treated and Ctr groups (Fig. 12). These data indicated that mannose may not modify gut permeability.

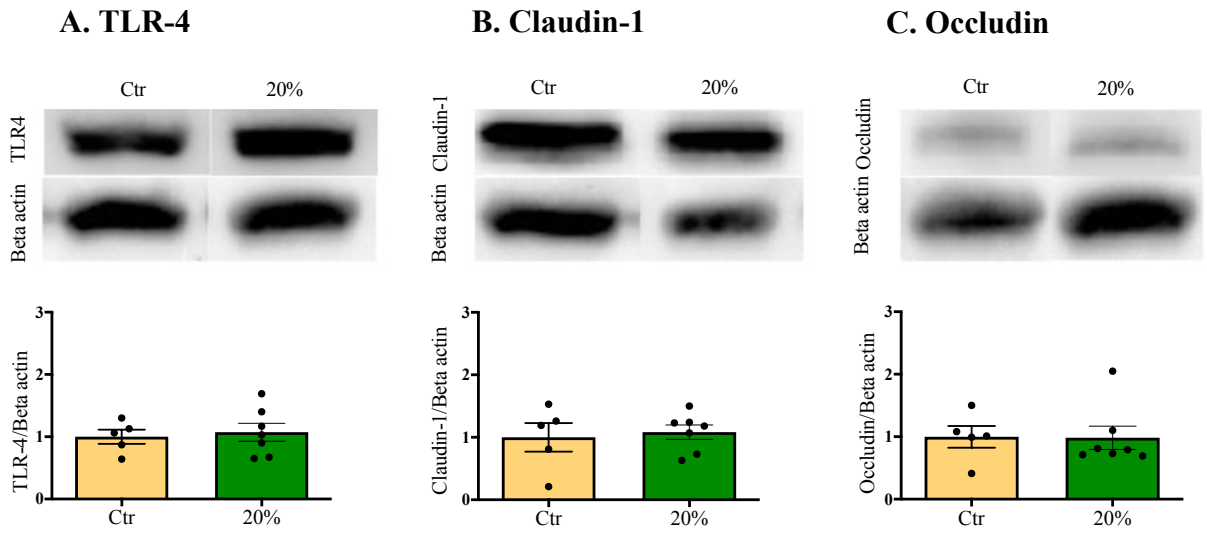


Figure 12. ApoE$^{-/-}$ mice treated with HFD supplemented orally with 0 (Ctr) or 20% mannose for 9 weeks. TLR4, claudin and occludin-1 expression levels were measured by western blotting on the intestine. Quantification graphs of TLR4 (A), claudin(B) and occludin-1 (C) and Beta-actin ratios expressed as fold changes. n=4-7.

4.5 Oral mannose decreases small peritoneal macrophage proportions and recruitment in the peritoneal cavity.

We next investigated macrophages, Small peritoneal macrophages (SPM) and large peritoneal macrophages (LPM), present in the peritoneal cavity described on page 18. Flow

cytometry showed a significant decrease of the pro-inflammatory SPM F4/80^{LO} macrophages, described previously, among CD11B+Ly6G- cells in 5% (3.0±0.3) and 20% (7.5±2.8) supplemented mice compared with Ctr (1.4±1.1). However, no difference in proportions of the resident LPM F4/80^{HI} macrophages was observed (Fig.13A,B). To determine macrophage recruitment to the PerC, upon 4 weeks of HFD mice were injected with 4% thioglycolate to create inflammation. Accordingly, SPMs were significantly decreased in 20% (49.0±8.3) mannose-supplemented mice compared to Ctr (74.0±1.3) (Fig.13C). In line with what is reported in the literature[127]. LPMs (F4/80^{HI}) were absent after thioglycolate stimulation.

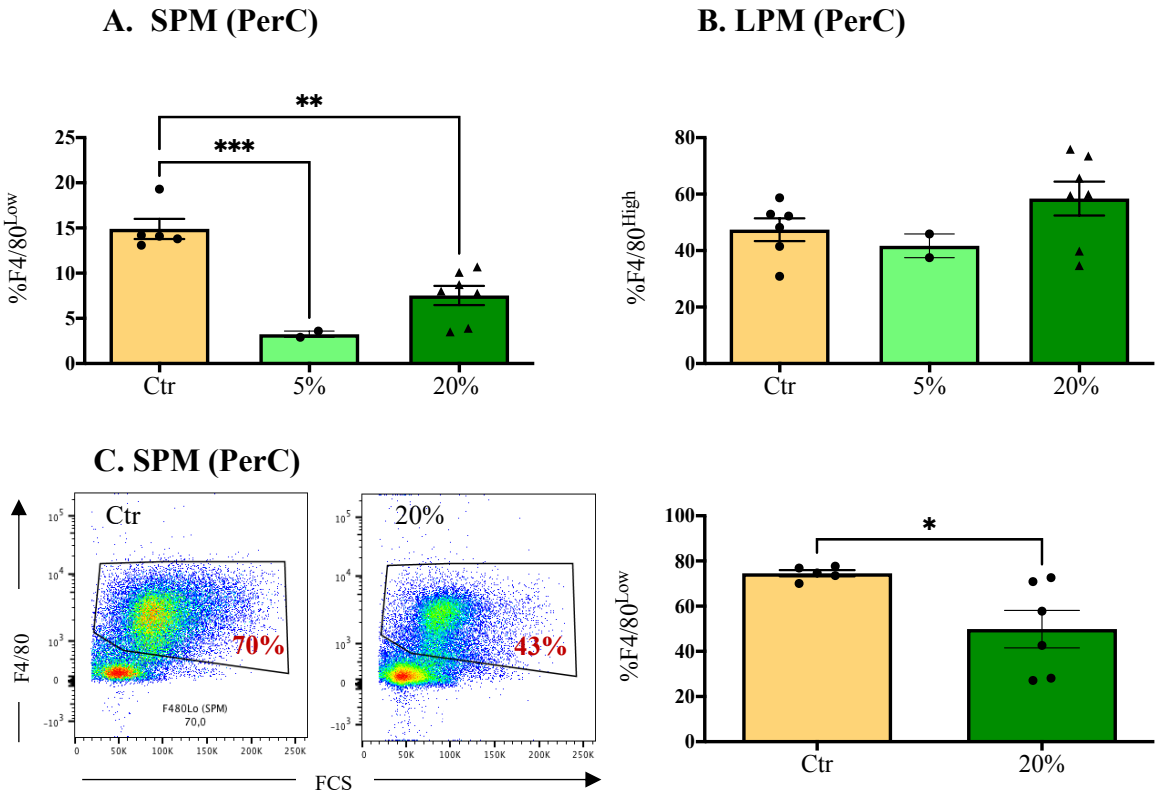


Figure 13. ApoE$^{-/-}$ mice treated with a HFD supplemented orally with 0 (Ctr), 5 or 20% mannose for 9 weeks. Peritoneal cavity (PerC) cells were collected. Flow cytometry quantifications of F4/80^{LO} SPMs (A) and F4/80^{HI} LPMs (B) from (CD11b+Ly6G-) cells. (C) ApoE$^{-/-}$ mice treated with a HFD supplemented orally with 0 or 20% mannose for 4 weeks. 4% Thioglycolate was injected and PerC cells were collected 4 days later. Representative flow cytometry dot plots with corresponding quantifications of F4/80^{LO} SPM. *P<0.05, **P<0.01, ***P<0.01, n=2-7. Data are mean ± SEM.

4.6 Oral mannose reduces proportions of circulating Ly6C-expressing monocytes.

Most of macrophages in the tissues migrate from circulating monocytes. We evaluated effects of mannose on monocytes and questioned if it affected myelopoiesis. First, proportions of circulating immune cells were measured. We observed no difference of blood monocytes and lymphocytes and a significant reduction of granulocytes in 20% (47±2) supplemented mice as compared with Ctr (56±2) (Fig.14A-C)

Next, blood and bone marrow monocyte subtypes as well as precursors were measured by flow cytometry in mice on a HFD supplemented with 5 or 20% mannose for 9 weeks. Blood Ly6C $^{LO/HI}$ ratio increased significantly in mice supplemented with 20% (1.8± 0.3) mannose compared to Ctr (0.8±0.1) reflecting a decrease in Ly6C^{HI} and an increase in Ly6C^{LO} (Fig.14D). Interestingly, Ly6C$^{LO/HI}$ ratio as well as (c-Kit+Sca1-) progenitor cells were also decreased in the bone marrow in mice supplemented with mannose 20% (Fig.14E,F).

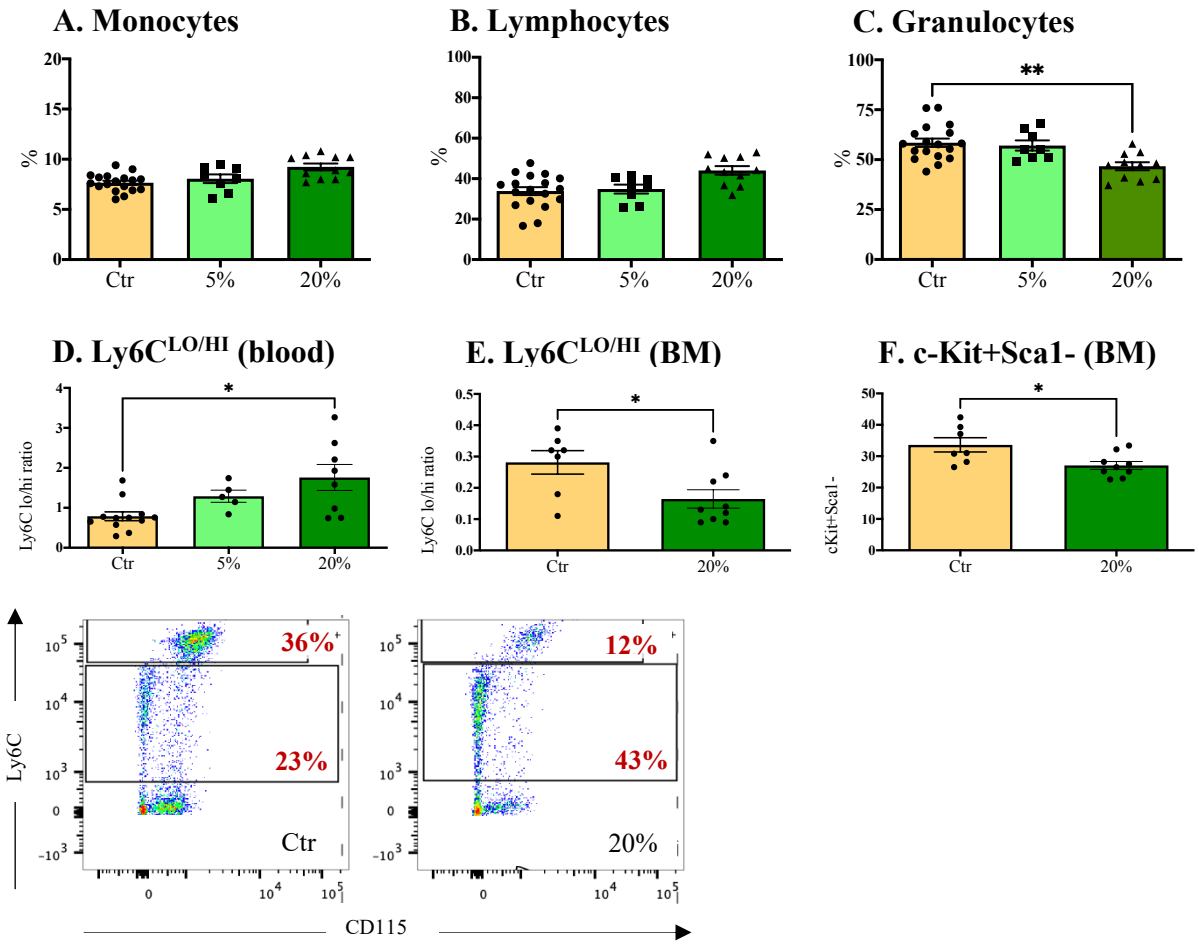


Figure 14. ApoE$^{-/-}$ mice treated with a HFD supplemented orally with 0 (Ctr), 5 or 20% mannose for 9 weeks. Blood was collected and immune cells were measured with vet ABC. Graphs represent proportions of Monocytes (A) lymphocytes (B) and granulocytes (C). Bone marrow (BM) cells were collected. Flow cytometry (D)Blood with representative dot plots and (E,F)BM and quantifications of Ly6C$^{LO/HI}$ ratio from CD11b+ly6G- cells vs 0% (Ctr). *P<0.05 **P<0.01, n=5-18. Data are mean ± SEM.

4.7 Oral mannose reduced atherosclerotic plaque development

We next assessed atherosclerotic plaques in the aortic sinus and the brachiocephalic artery. OilRedO staining showed a significant reduction in plaque size in mice supplemented with 5 and 20% mannose compared with 0% (Ctr) in both segments (Fig.15). Aortic sinus showed a decrease by 41% in both 5 (0.23±.03) and 20% (0.23±0.04) mannose compared to Ctl (0.40±.003). BCA decreased by 45% in 5 (0.05±.01) and 63% in 20% (0.03±.01) compared to Ctl (0.08±.01).

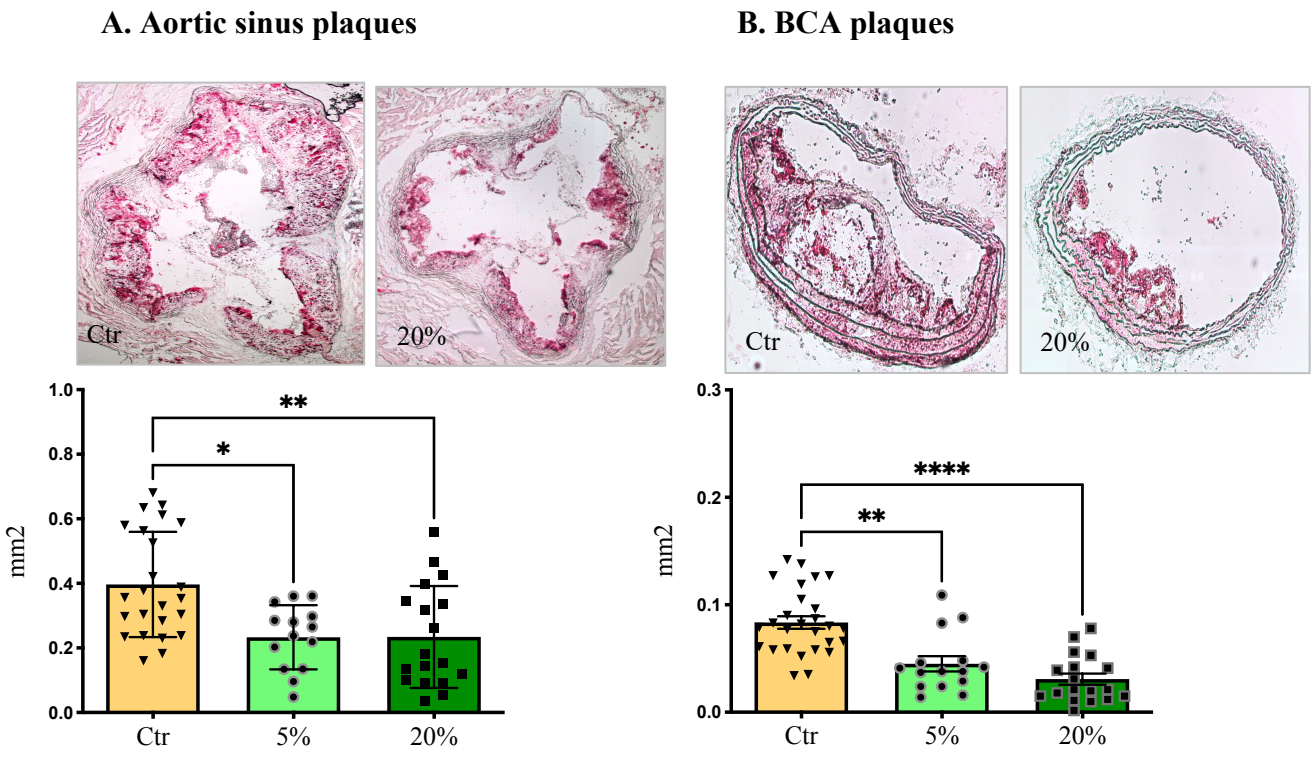


Figure 15. ApoE$^{-/-}$ mice treated with a HFD supplemented with 0 (Ctr), 5 or 20% mannose for 9 weeks. Representative OilRedO images and plaque size quantifications at the aortic sinus (A) and

at the brachiocephalic artery (BCA) (B). *P<0.05, **P<0.01, ***P<0.001, n=15-24. Data are mean ± SEM.

4.8 Oral mannose supplementation increased atherosclerotic plaque stability.

We next analysed plaque composition and stability using immunohistochemistry. Smooth muscle alpha actin (α-SMA, marker of smooth muscle cells), was increased significantly by 42% within the atherosclerotic plaque in 20% (1.4±0.2) mannose supplemented mice as compared to controls (0.1±0.1) (Fig.16A,B). In contrast, no difference in CD68-expressing macrophages within the aortic sinus plaque was observed (Fig.16A,C). Plaque stability index was calculated by considering the proportion of VSMCs to NC within plaques. Masson's trichrome staining on aortic sinuses was performed to quantify plaque necrotic core (Fig. 16D). The ratio of α-SMA and NCs was significantly increased in mice supplemented with 20% (1.4±0.1) mannose compared to Ctr (0.7±0.2).

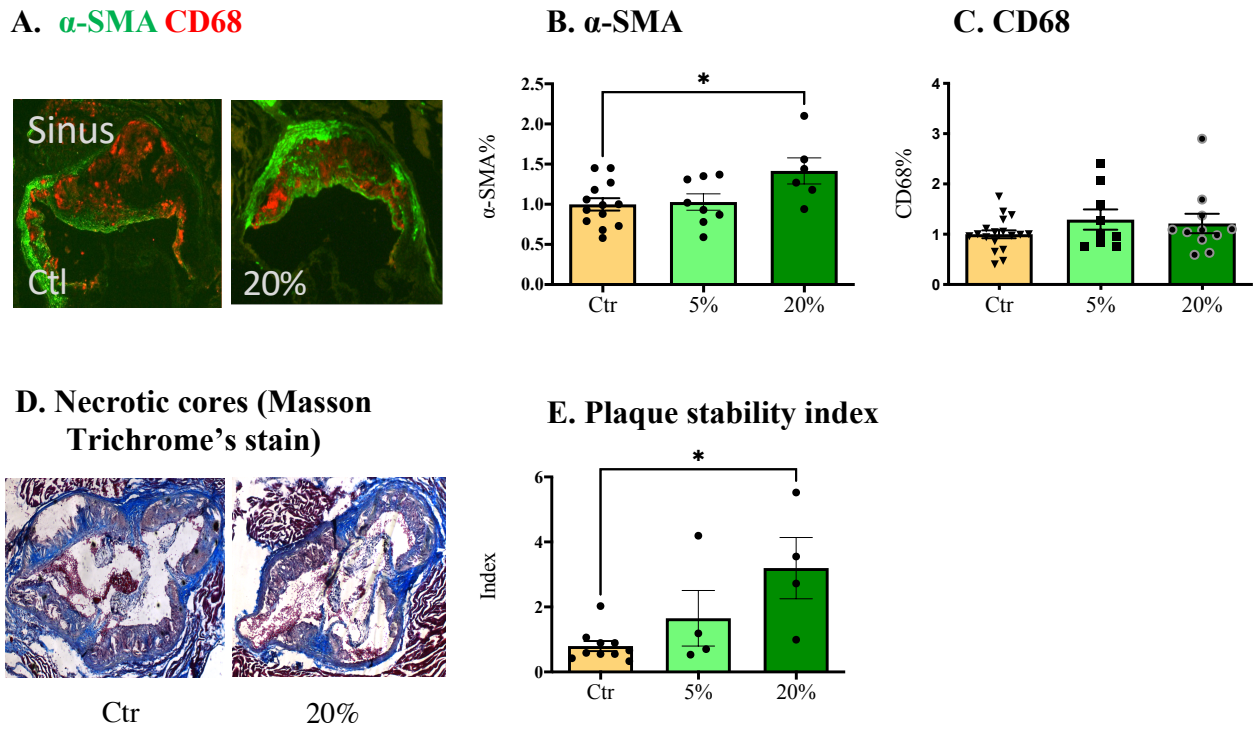


Figure 16. ApoE$^{-/-}$ mice treated with a HFD supplemented orally with 0 (Ctr), 5 or 20% mannose for 9 weeks. Plaque composition and stability index were assessed. Representative immunofluorescence images and quantification graphs of α-SMA (A,B) and CD68 (A,C) in the aortic sinus. Representative images of Masson's Trichrome staining (D) and plaque stability as the ratio of α-SMA and necrotic core (E). *P< 0.05, n=4-18. Data are mean ± SEM.

4.9 Mannose supplementation by IP injections did not change atherosclerotic plaque development.

We next questioned whether the atheroprotective effect of mannose is through regulation of gut microbiota. We evaluated atherosclerosis in mannose supplementation by IP injections to bypass the gut. ApoE$^{-/-}$ mice received a HFD with 5g/kg mannose injected intraperitoneally 5 times a week. There were no differences in HFD-induced of body weight and lipid levels (Fig.17A-C). Interestingly, no changes in plaque size were observed between mannose supplemented and control (PBS) groups in the aortic sinus or the brachiocephalic artery (Fig.17 D,E). This result suggests that there could be an involvement of gut microbiota in the atheroprotective effect of mannose.

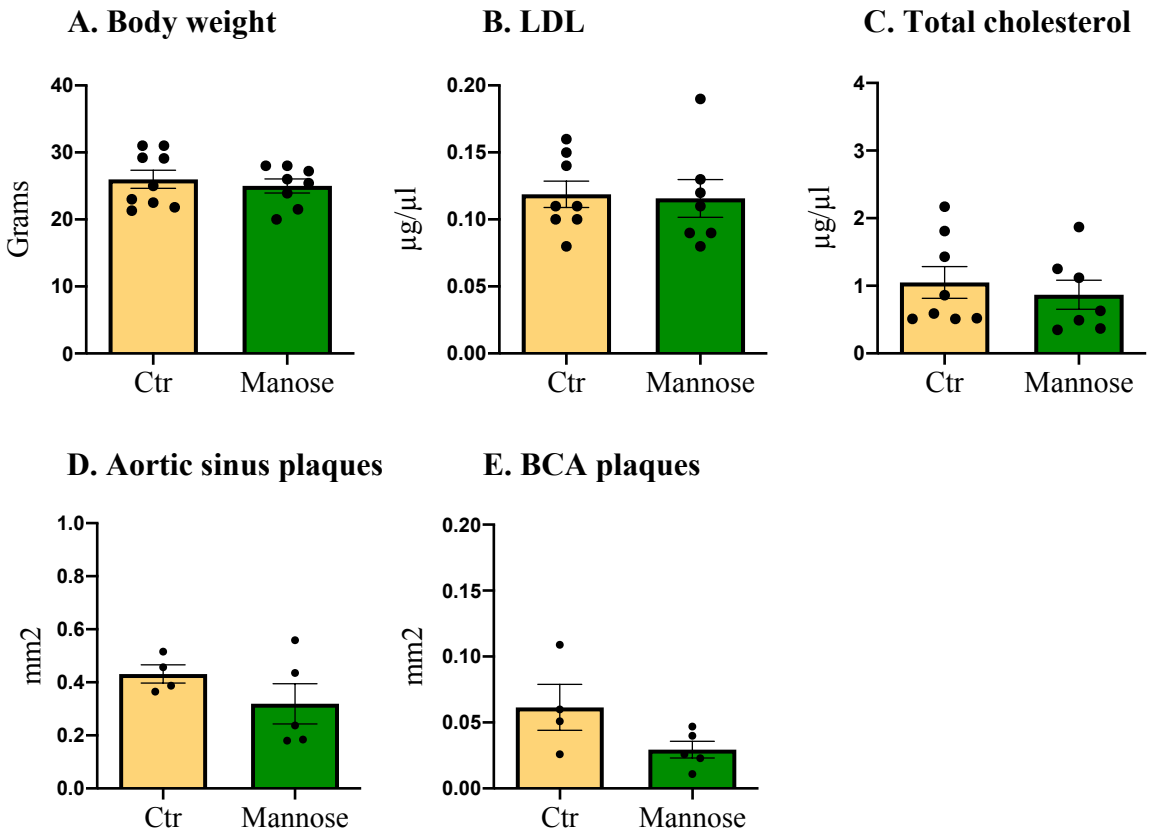


Figure 17. ApoE$^{-/-}$ mice treated with HFD supplemented with 0 (PBS) or 5g/kg mannose by IP injections for 9 weeks. Animal parameters (A) body weight, (grams). (B) plasma LDL levels (µg/µl). (C) plasma total cholesterol levels (µg/µl). (D,E) Representative OilredO staining images and plaque size quantifications at the aortic sinus and at the brachiocephalic artery. n=4-8. Data are mean ± SEM.

5. Discussion

In this study, we investigated how mannose supplementation to HFD regulates the hosts different systems including the gut microbiota composition and/or the inflammatory response in the bone marrow, the peritoneal cavity and the intestine leading to atherosclerotic plaque development. We observed that mannose supplementation decreased atherosclerotic plaque size in mice under HFD conditions when mannose is given orally however, these effects were not observed when it is supplemented by IP injections, bypassing the gut. This suggests that the protective effect of mannose could be due to the modification of gut microbiota bacterial composition leading to an anti-inflammatory response related to the regulation of monocytes/macrophages. In humans, clinical evidences largely demonstrated that high consumption of sugars is a risk factor in developing obesity and metabolic syndrome which are important risk factors for cardiovascular diseases [341]. In rodents there are also several data reporting the effects of different sugar supplementations on body weight and cholesterol levels under HFD conditions. Increase in weight and plasma cholesterol levels were found in swiss mice with fructose-water for 9 weeks [342] and in hamsters fed HF and high fructose diet for 14 days [343]. On the contrary, rats on a HFD supplemented with fructose or glucose for 20 weeks showed no difference in body weight and a decrease of total cholesterol levels compared with HFD controls [299]. However, treatment of wild type mice on a HFD with sucrose for 15 weeks did not show any difference in body weight and plasma cholesterol levels [344] and an $ApoE^{-/-}$ mice group on a high fructose diet reported no changes in cholesterol levels, but increased atherosclerotic lesions compared to control mice [345]. In our study we showed that mannose supplementation in $ApoE^{-/-}$ mice under HFD did not change the increase of body weight and plasma lipid levels as compared with the control group. Accordingly, Sharma and coauthors also did not see any effects of orally supplemented mannose in old C57BL/6 mice [330]. Indeed, atheroprone $ApoE^{-/-}$ mice have increased basal cholesterol levels as compared with non-genetically modified mice, which could explain in part the opposing results on lipid levels obtained with similar treatments. Importantly, the current treatment to reduce CAD caused by atherosclerosis is lipid lowering drugs. Interestingly, we demonstrated that oral treatment of $ApoE^{-/-}$ mice with mannose decreased atherosclerotic plaques under HFD conditions without changes in lipid levels suggesting that mannose prevents atherosclerosis through a different process. We also observed an increase in

plasma mannose levels in our mice. However, two studies revealed that increased plasma mannose levels were associated with incident type 2 diabetes mellitus and cardiovascular diseases [346, 347]. This could be explained as part of the impossibility of glucose or mannose to enter in the cell due to a reduced expression of GLUT4, the primary glucose and mannose carrier in the adult [347], during insulin resistance states, leading to an increased level of these sugars in the plasma. Increased mannose levels in patients with type 2 diabetes is a consequence of the physiopathology of the disease. Nevertheless, any dietary sugars, including mannose, should be avoided by pre-diabetic or diabetic individuals. Also, plasma mannose levels were increased in mice supplemented with 5% compared with Ctr. The absence of a significant increase in 20% supplemented mice could be related to a low number of mice in this group. Currently, several studies exhibit a relation between the gut dysbiosis and atherosclerosis [348-350]. In our study the protective effect of oral mannose in plaque formation could be a consequence of the change in the composition of the gut microbiota. The Firmicutes/Bacteroidetes ratio is considered to be an indicator for gut dysbiosis. Its rise has been reported in different human and mice studies of obesity [210, 351-353], hypertension [354], increase of lipids [355-357], endotoxemia [358, 359], coronary artery disease [360, 361] and myocarditis [362]. Lower abundance of Bacteroides have been found in humans with CAD [363]. Horne, et al demonstrated that HF and high fructose diet increased the Firmicute/Bacteroidetes ratio[343]. Increased Bacteroidetes to Firmicutes ratio in the gut microbiota was exposed by a study demonstrating that mannose supplementation prevented HFD mice obesity and inflammation [330]. In line with these reports, here we demonstrate here that oral mannose supplementation prevents the increase of this ratio caused by the HFD, promoting the increased number in the Bacteroidetes and lowering the Firmicutes. In addition, when mannose was supplemented by IP injections, atherosclerotic plaques were unchanged. Microbiota is hypothesized to be unaffected by the IP administration of mannose, eluding contact with the digestive tract. Therefore, this result suggest that the effect of mannose is possibly through regulation of gut microbiota composition.

To test the gut dysbiosis inflammation we measured the LPS receptor, TLR4 expression, considered to be involved in the consumption of dietary fats and metabolic inflammation. TLR4-deficient mice on a HFD for 8 weeks with increased Firmicutes/Bacteroidetes ratio showed a reduction of pro-inflammatory cytokines in the epididymal fat [364]. Also, inflammation increases

gut permeability by decreasing tight junctions. High intake of fructose in rats impairs intestinal barrier function due to the decrease of the expression of occluding tight junction protein [365]. ApoE-/- mice on a HFD treated with polyphenols or Akkermansia muciniphila had a reduction in circulating endotoxin level due to increased intestinal tight junctions, occludin and claudin-1 [366, 367]. In our study, expression of TLR-4 on macrophages of the LP of the small intestine was reduced in mice supplemented with mannose as compared with controls. in contrast, western blotting on the intestinal samples did not show changes in TLR4, or the tight junctions claudin-1 and occludin expression levels compared with controls.

Analysis of gut microbiota in patients with CAD shows a relative depletion of Bacteroidetes compared with controls without CAD with coronary risk factors [368]. Accordingly, it has been shown that ApoE-/- mice gavaged with live Bacteriodetes decreased plasma LPS levels and atherosclerotic lesion formation [363]. Consistently, in our study mannose supplementation of mice prevented the increase of plasma LPS levels observed in the HFD control group, suggesting that mannose regulates gut microbiota, by diminishing bacteria products that cause gut inflammation.

To further explore the systemic changes induced by HFD inflammation, we observed two populations of macrophages in the PerC: an abundant population of so-called “large” peritoneal macrophages (LPM) that are embryonically seeded and long-lived, and a rarer population of short-lived MHCII+ monocyte-derived cells termed small peritoneal macrophages (SPM)[369]. It is already known that mice SPM cells are upregulated following a thioglycolate injection, making LPM phenotype cells barely detectable [127]. In our study, after inducing inflammation with thioglycolate, all mice groups lost the LPMs. Nevertheless, mannose reduced peritoneal SPMs recruitment compared with control group, probably by reducing the flux of blood monocytes and controlling the differentiation of these cells in the PerC. In absence of thioglycolate, we observed both populations showing a decrease in the SPM and no changes in the LPM. A lower recruitment of SPM cells confirms mannose ability to supress thioglycolate-elicited inflammatory response.

Yang et al established in ApoE-/- mice under HFD that blood $Ly6C^{HI}$ monocytes are associated with pro-inflammatory/atherogenic function and that $Ly6C^{LO}$ subtypes present anti-

inflammatory/atherogenic features [370]. Also, some drugs such as, pravastatin suppresses atherosclerosis development by inhibiting $Ly6C^{HI}$ cell expression in ApoE-/- mice [371]. Mannose treatment in our mice increased the $Ly6C^{LO/HI}$ ratio compared to controls, preventing a major inflammatory component in the atherosclerotic plaque formation. The opposite effect was observed in the BM, suggesting that mannose retains $Ly6C^{HI}$ monocytes in the BM, leading to a decrease of their number in the circulation. BM progenitor cell markers c-Kit+Sca1- were reduced in mannose treated mice, indicating that mannose seems to accelerate BM cell differentiation. Interestingly blood cell count indicated a reduction of granulocytes in mice treated with mannose and no difference in monocytes. ApoE polymorphism in humans is not associated with changes in the white blood cell count [372].

Importantly, mannose supplementation decreased atherosclerotic plaque size in both aortic sinus and BCA. Supplementation with berberine, main active component of an ancient Chinese herb Coptis chinensis french, also showed a reduction of atherosclerosis through gut microbiota (Firmicutes and Verrucomicrobia) in ApoE-/- mice under HFD [373]. However, berberine had to be administered intragrastrically because of its poor oral bioavailability, and it did not influence plaque stability. We found that mannose increased plaque stability by increasing smooth muscle cells within the plaque. Lineage-tracing studies demonstrated that atherosclerotic plaque stability depends on the thickness and composition of the fibrous cap [374]. Indeed, the fibrous cap predominately contains cells derived from VSMCs that are the primary source of collagen, providing mechanical tensile strength and resistance to rupture [375]. Collagen VIII deficiency in ApoE-/- mice showed a thinning of the fibrous cap due to decreased SMC proliferation and migration [376]. Vulnerable plaques and ruptured plaques show large necrotic cores (NCs) with or without plaque hemorrhage and a thin fibrous cap [186]. In our study mannose increased plaque SMC content and and plaque stability index taking in account plaque NCs (ratio of α-SMA positive cells to NC size) compared with controls. No differences in CD68+ macrophages were observed. However, we should keep in mind that the atherosclerotic plaque areas were smaller in mannose treated mice, then proportionally there should be less plaque macrophage content compared with controls.

Our study has some limitations. Mannose supplementation was ad libitum by water bottles which makes the exact drink intake inaccurate. The metabolism of murine models is different from

humans, mannose supplementation in mouse does not translate exactly into humans. Mice engage in coprophagia, which means they re-ingest their feces, this may influence the microbiota and the observed results.

Histomorphology remains a powerful routine evaluating intestinal inflammation in animal models. Evaluating quality and dimension of inflammatory cell infiltrates, epithelial changes and overall mucosal architecture would give more information of histopathologic changes of the gut. In our study IP injections of mannose did not change atherosclerotic plaque development. However, mannose plasma levels were measured only in mice supplemented orally with mannose.

Bacteria outside of the gut can be important contributors to immune responses. Recent studies have established the presence of bacteria in atherosclerotic plaques and suggested their possible contribution to the development of cardiovascular diseases [377-379]. Rabbits on high cholesterol diet infected with C. Pneumoniae and treated with antibiotic had an increase of atherosclerosis compared to untreated controls [380]. 16S sequencing of plaque samples from patients with atherosclerosis detected Proteobacteria (90.5%), Actinobacteria (5.3%), Bacteroidetes (1.2%) and Firmicutes (1.1%). A correlation between Granulicatella and an increased total cholesterol concentration was described [381]. Due to plaque size in mice, we could not detect any bacteria within the plaques of our mice.

Mannose prevents the proatherogenic effects caused by HFD, however excessive consumption of diets high in sugars, meats and saturated fat, frequently known as western diet, may lead to obesity and metabolic syndrome [244, 283]. Meats are rich in L-Carnitine and it is an abundant component of the western diet commonly implicated in CVD [283]. L-Carnitine is metabolized by gut microbiota in Trimethylamine (TMA) and then oxidized into TMAO in the liver [382]. TMAO and its precursors have been demonstrated to be involved in CVD and atherosclerosis [283, 372, 383]. Supplementing HFD with L-carnitine would be a high-grade inflammatory model to investigate the effect of mannose in atherosclerosis. Next we will measure gut microbiota derived metabolites in plasma such as short-chain fatty acids (SCFAs) (acetic acid, propionic acid, butyric acid) that have been shown to play a positive role in cardiovascular system. In contrast TMAO and secondary bile acids (deoxycholic acid or lithocholic acid) contribute to inflammation and atherosclerotic burden[384].

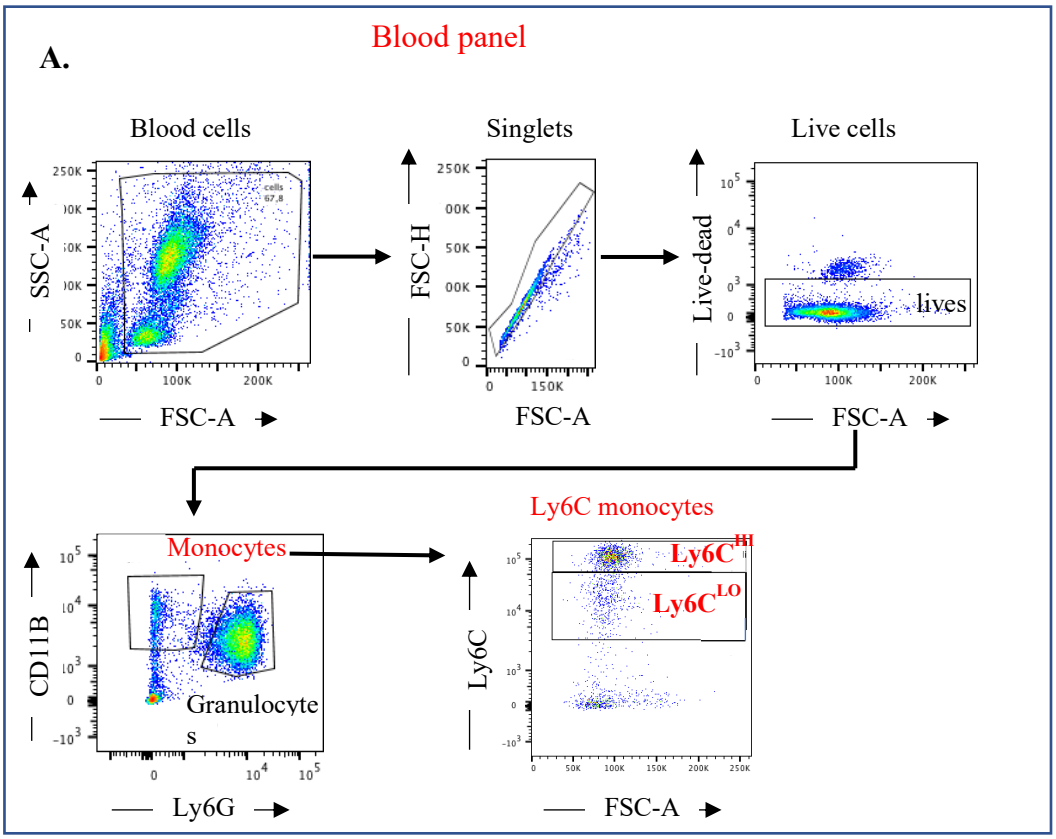
Circulating Ly6C$^{HI/LO}$ monocyte ratio was regulated by mannose, therefore measuring plasma chemokines such as, CCL2, CLL3, CCL4, CX3CL1, would inform us about the role of mannose on circulating inflammatory cells which ultimately leads to atherosclerosis.

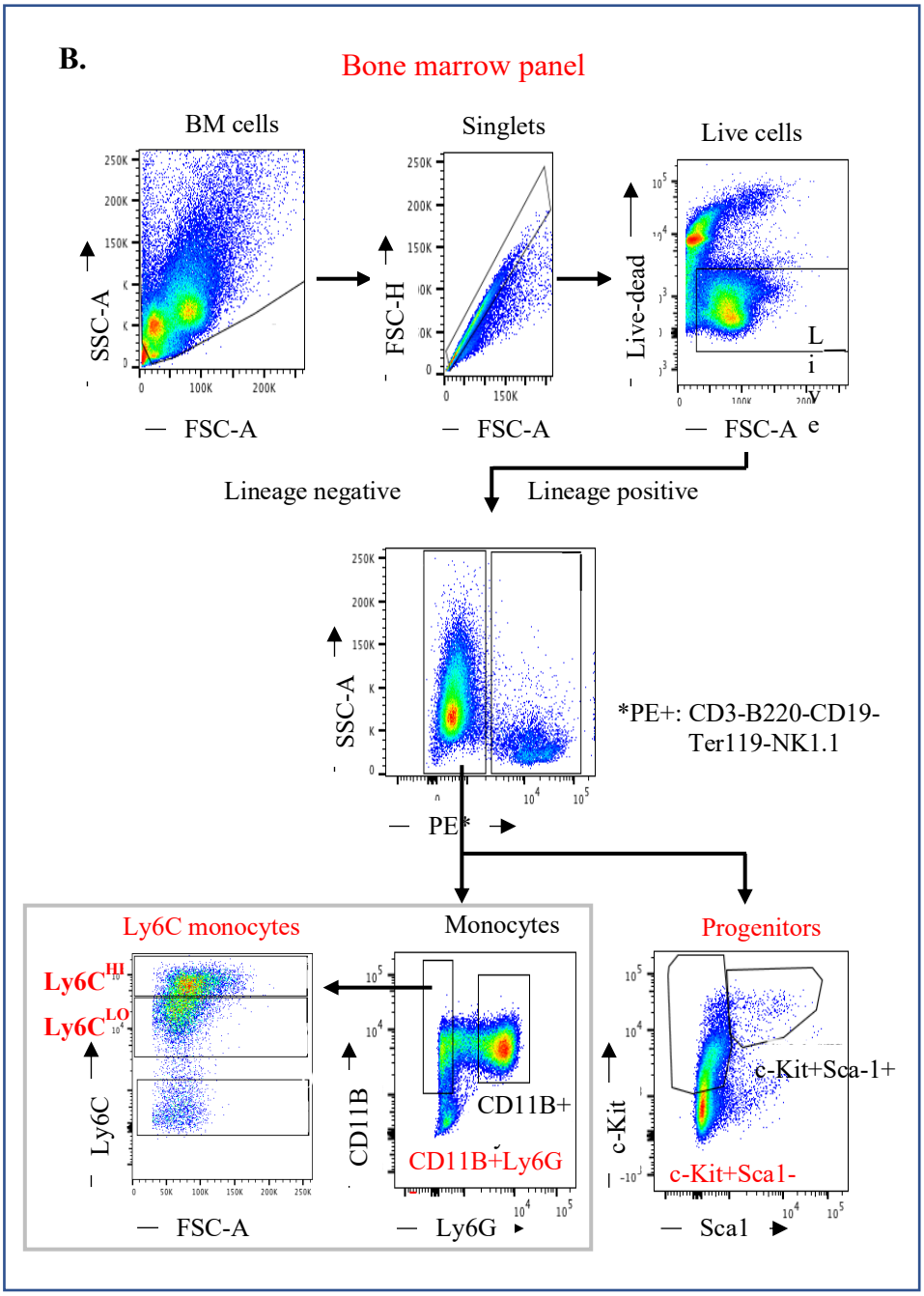
To further analyze gut permeability and its relationship with inflammation we plan to measure fecal mannose and endotoxin levels. Confirming the atheroprotective effect of mannose through gut microbiota regulation, we will realize microbiota transplantation, also known as fecal trasnsplants experiments, where microbiota of mannose treated mice will be transplanted into HFD germ free mice to see if similar effects as mannose oral supplementation are observed in plaque development. Also, to evaluate the effects of a different sugar diet on atherosclerosis ApoE-/- mice will be treated with glucose (epimer of mannose) under HFD.

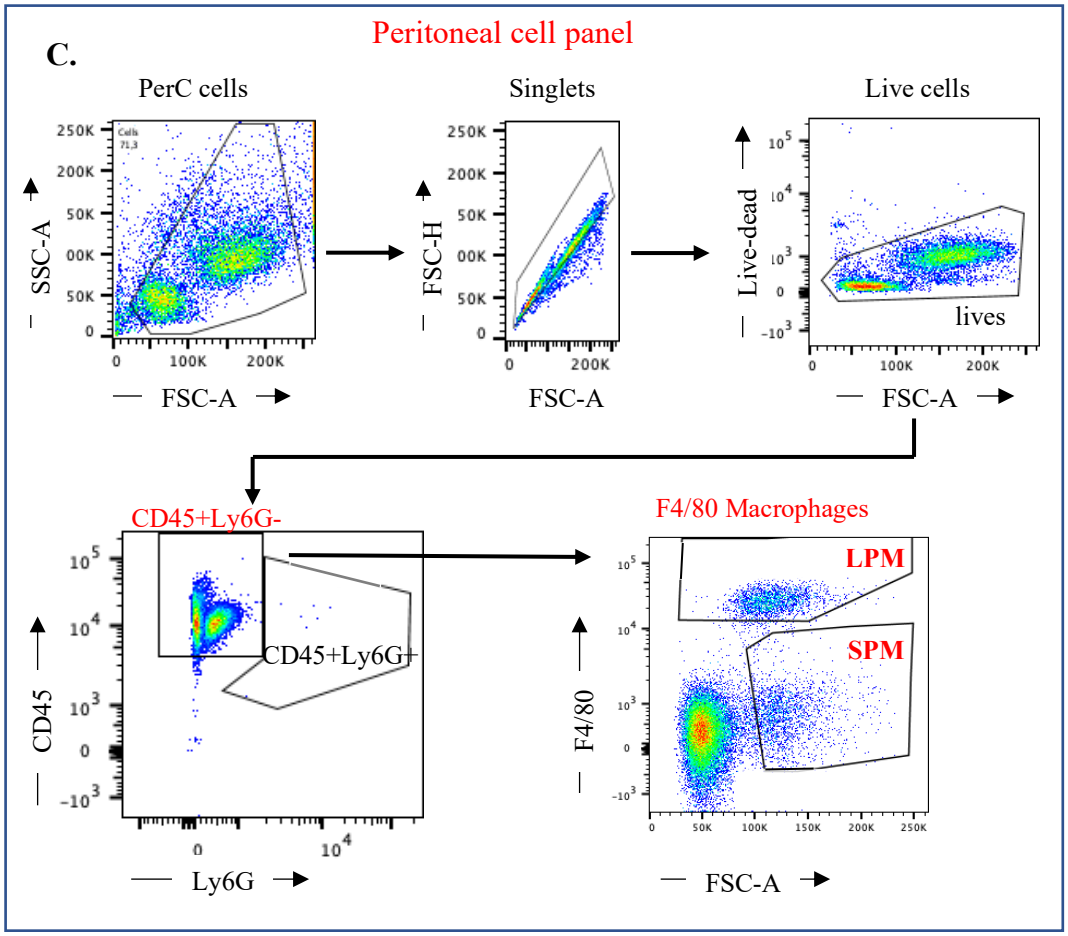
6. Conclusions

Under HFD conditions oral, mannose sugar has the potential to regulate gut microbiota, restoring the Firmicutes/Bacteroidetes ratio, which is disbalanced by HFD. In this way, mannose keeps a microbiota more similar to that seen in chow diet fed mice by diminishing gut bacterial products that cause inflammation, less intestinal macrophages are found and activated in the gut, these cells display decreased the expression of TLR4, and the increase of LPS in plasma is prevented, even though mannose did not regulate tight junctions in the intestine or improve the gut barrier function. Oral mannose regulates inflammation by reducing SPM proportions and differentiation, which appear to be the major source of inflammatory mediators in PerC during infection presenting a pro-inflammatory functional profile, without noticing changes in the LPM resident macrophages. A similar effect was observed when inflammation was induced by thioglycolate. Mannose by reducing pro-inflammatory macrophages, could also reduce pro-inflammatory cytokines such as IL-1, TNF, and other mediators [385] that are involved in the atherosclerosis development. Mannose increases circulating $Ly6C^{LO/HI}$ monocytes which means that it regulates the proinflammatory process. This may also mean that mannose will favour the differentiation of macrophages towards an M2 phenotype rather than M1. Measuring circulating cytokines and macrophage subtypes will be performed. Mannose raises the retention of $Ly6C^{LO/HI}$ in the BM and affects myelopoiesis by diminishing progenitors c-Kit+Sca1- markers, demonstrating that regulates cell differentiation in the BM and consequently reducing the proinflammatory cells in the circulation and inflammatory response that contribute to atherosclerosis progression. Oral mannose supplementation, most importantly, reduces atherosclerotic lesions, and increases plaque stability, without lowering the lipids levels in the plasma through regulation of gut microbiota. Whereas IP administration of mannose, which bypasses the gut, does not show any plaque reduction. Lipid lowering drugs such as statins are a common medication prescribed to prevent CVD and atherosclerosis. However, statins could have severe side effects in some patients [386, 387]. Long-term treatment with mannose in humans and mice has been shown to be effective and safe [388, 389]. Modulation of gut microbiota by replacing some dietary sugars with mannose may yield an easily applicable and cost-effective strategy that could provide an adjunct to current regimes to treat or prevent atherosclerosis.

Supplemental Figure




B.
Bone marrow panel
BM cells
Singlets
Live cells
SSC-A
FSC-A
FSC-H
FSC-A
Live-dead
FSC-A
Lineage negative
Lineage positive
SSC-A
PE*
*PE+: CD3-B220-CD19-
Ter119-NK1.1
Ly6C monocytes
Monocytes
Progenitors
Ly6C^HI
Ly6C^LO
Ly6C
FSC-A
CD11B
CD11B+
CD11B+Ly6G
Ly6G
c-Kit
c-Kit+Sca-1+
c-Kit+Sca1-
Sca1



Supplemental figure 1. Gating strategies for flowcytometry analysis. A. Blood. B. Bone marrow. C. Peritoneal cavity (PerC) panel.

7. References

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