THE EFFECT OF MELANOCORTIN-1 RECEPTOR DEFICIENCY ON CHOLESTEROL TRANSPORTER GENES IN ATHEROSCLEROTIC APOE KNOCKOUT MOUSE MODEL

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The melanocortin system comprises of melanocortin peptides; α-, β- and γ-melanocortin stimulating hormones (MSH) and adrenocorticotropic hormone (ACTH) derived from the proteolytic degradation of pro-opiomelanocortin (POMC) prohormone. These hormones regulate important physiological processes such as skin pigmentation, energy expenditure and food intake as well as exocrine and inflammatory activities amongst other functions. Biological activities of melanocortins occur via the activation of five melanocortin receptors (MC1 – MC5). MC1 receptor is activated by α-MSH and plays an important role in inflammatory activities. Recent studies revealed that the activation of MC1-R in macrophages reduces cholesterol uptake and promotes cholesterol efflux. Against this background, we hypothesised that MC1-R deficiency leads to decreased expression of reverse cholesterol transport (RCT) genes and consequently, impact the process of RCT. In this study, we investigated the transcriptional regulation of cholesterol transporter genes (*ABCA1, ABCG1*, *ABCG5, ABCG8, SR-B1, SR-A1 and CD36*) in wild type (WT) and MC1 receptor deficient (MC1-$R^{e/e}$) mice on ApoE knockout background as well as the effect of high fat diet (4 weeks) in the two genotypes. The RNA from aorta, liver, spleen and bone marrow of the mice was extracted, converted to cDNA and then quantified with real-time polymerase chain reaction (qPCR) for the selected genes. We also conducted *in vivo* cholesterol efflux assay by loading tritiated cholesterol into bone marrow-derived macrophages and intraperitoneally injected them into WT and MC1-$R^{e/e}$ mice. Twenty-four to forty-eight hours after the injection, radioactivity accumulation of H^3-cholesterol in the plasma, liver and feces was measured. We observed that crucial cholesterol transporter genes; *ABCA1* and *ABCG1* were downregulated in the aorta of MC1-$R^{e/e}$ mice, *ABCG5* and *ABCG8* expression in the liver of this group of mice were also reduced, an effect exacerbated by diet rich-in high fat. Generally, the cytokines and adhesion molecules (*IL-1β, IL-6, CCL-2, CD62P* etc.) analysed from the aorta of the MC1-$R^{e/e}$ mice were significantly reduced compared to WT mice. The cholesterol efflux assay showed no major differences between the genotypes in terms of cholesterol efflux capacity. In conclusion, MC1-R deficiency in ApoE knockout mice resulted in reduced expression of RCT genes particularly in the aorta and liver, which might hamper the efficiency of RCT process.

Table of Contents

ABSTRACT

1. INTRODUCTION 1

1.1 Cardiovascular Disease and Atherosclerosis 1

1.2 Cholesterol and Cholesterol Carriers 4

1.3 Cholesterol Efflux and Reverse Cholesterol Transporters 6

1.4 Melanocortins and the Melanocortin System 8

2. RESULTS 11

2.1 ABCA1 and ABCG1 Cholesterol Transporters are Down-regulated in the Aorta of MC1-R Deficient Mice 11

2.2 Deficiency in MC1-R Resulted in Reduced ABCG5 and ABCG8 Expression in the Liver of HFD-fed Mice 15

2.3 Cholesterol-Rich Diet Influence the Expression of Cholesterol Transporter Genes and Pro-inflammatory mediators in the Spleen and Bone Marrow of Mice 16

2.4 H^3-Cholesterol Absorption into Plasma, Liver and Excretion in Feces of Mice via Cholesterol Efflux Mechanism 18

3. DISCUSSION 20

4. MATERIALS AND METHODS 25

4.1 Animals and Tissue Extraction 25

4.2 RNA Extraction 25

4.3 cDNA Conversion 26

4.4 Quantitative RT-PCR 26

4.5.1 Macrophage Cell Culture and ^{3}H-Cholesterol Labelling 27

4.6 Radioactivity Measurements with Scintillation Counter 27

4.6.1 Plasma 27

4.6.2 Liver 28

4.6.3 Feces 28

4.7 Statistics 28

5. ACKNOWLEDGEMENTS **29**

6. ABBREVIATIONS LIST **29**

7. REFERENCE **32**

8. APPENDICES **35**

8.1 Supplemental Fig. 1: Cholesterol Transporters and Cytokines Gene in the Spleen of Mice 35

8.2 Supplemental Fig. 2: Cholesterol Transporters and Scavenger Receptors in the Bone Marrow 36

1. INTRODUCTION

1.1 Cardiovascular Disease and Atherosclerosis

Cardiovascular diseases (CVDs) are classes of disorders that affect the heart and blood vessels. It has been the leading cause of death over the past decade especially, ischaemic heart disease (IHD) and stroke. The development of CVDs can be genetically linked or as a result of infection. However, environmental factors such as increased intake of saturated fat and sedentary life style constitute a higher influence in the development of these conditions. In 2012, 31% (17.5 million people) of global mortality was caused by cardiovascular complications. IHD claimed 7.4 million people lives and 6.7 million deaths were associated with stroke. Thus, CVDs are the number one cause of death worldwide (WHO, 2016). In Europe, CVDs account for 4 million deaths each year. A prominent risk factor for the development of CVD is the luminal narrowing of the major arteries that supply blood to the brain (stroke), the heart (CHD - coronary heart disease) and the lower extremities (PVD - peripheral vascular disease). Stroke and myocardial infarction (heart attack) are the two main acute cardiovascular events that restrict blood flow to the brain and heart. This mostly stems from the build-up of fat deposits within the walls of the blood vessels – a process called atherosclerosis.

Atherosclerosis is one of the most common CVDs. It is currently recognised as a chronic inflammatory disease characterised by the narrowing of the lumen of arteries with fatty material, that eventually cause plaque (atheroma) build-up. Consequently, thrombosis ensues and the obstruction of blood supply to vital organs. The atheroma in atherosclerosis disease is a lipoprotein-driven condition and results in intimal inflammation, calcification, fibrosis and necrosis. The plaques also contain cellular debris as well as hydroxyapatite (calcium deposits), which in addition to the fatty deposits are very thrombogenic. Under stable conditions, the atheroma plaques are protected from the circulating blood by a thick fibrous cap made up of extracellular matrix proteins (e.g. collagen) and vascular smooth muscle cells (Libby *et al*; 2011). The fibrous cap may be degraded by zinc-dependent matrix metalloproteinases (MMPs) enzymes secreted by macrophages, which increases the risk of plaque rupture.

MMPs may also be responsible for the degradation of elastic laminae present in the layers of the arterial wall, thereby promoting the migration of cells of the smooth muscle from the tunica media to the fibrous cap and consequently, strengthening the plaque. A plaque can be characterised as stable or unstable based on the thickness of the fibrous cap and size of the lipid core. A stable plaque is less likely to rupture and it consists of fibrous cap rich in thick smooth muscle-cells with a small inner lipid core. On the other hand, vulnerable or unstable plaques with high susceptibility of rupturing contain large necrotic lipid core and thin fibrous cap (**Fig. 1**). A defective fibrous cap increases the risk of exposing the thrombogenic content of the plaque to the circulating blood – thrombosis. A large percentage of plaque ruptures is clinically silent and a very small fraction actuates stroke and myocardial infarction (Libby *et al*; 2011).

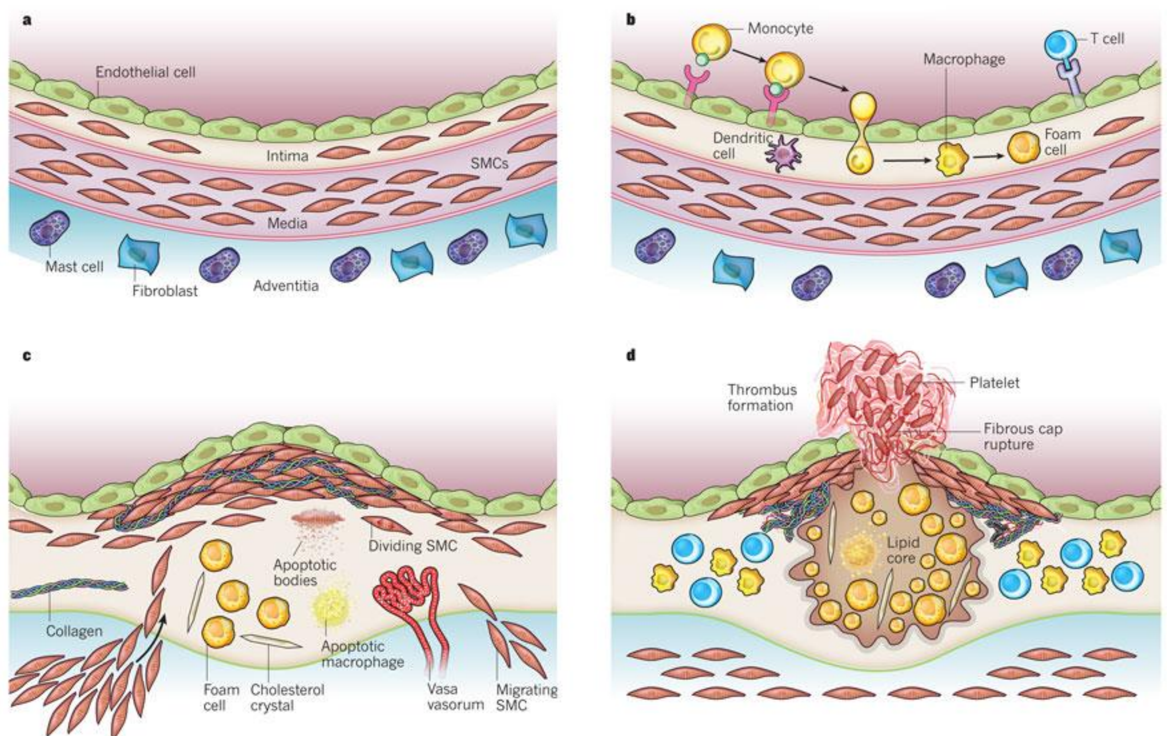

Fig. 1 Stages of atherosclerotic lesion development (Adapted from Libby *et al*; 2011)

The actual cause of atherosclerosis is unknown, but it is preceded by an initial process known as atherogenesis – a disorder of the arterial wall.

The cellular components of atherosclerosis include intimal smooth muscle cells, endothelial cells and leukocytes. These are major players in the development of this chronic immunoinflammatory disease of medium and large-sized arteries laden by lipid. Atherosclerotic lesion is initiated when the endothelium becomes leaky and dysfunctional, which may encourage the adherence of platelets, plasma and lipoprotein particles (Davies *et al*; 1988). Here, the trapped lipoprotein particles are oxidised into pro-inflammatory, pro-atherogenic, cytotoxic and chemotaxic entity, of which the pathophysiology is not fully understood yet. The process of atherogenesis involves the transmigration of monocytes via the endothelium into the intima layer of the arterial wall where they differentiate mainly into macrophages. These cells scavenge for modified lipoproteins such as oxidised low-density lipoprotein (oxLDL) turning them eventually into lipid-rich foam cells. The uptake of cholesterol by macrophages is facilitated by the expression of scavenger receptors such as *CD36*/FAT (fatty acid translocate) and *SR-A1* (scavenger receptor class A type 1). The transmigration process is promoted by inflamed endothelial cells expressing high levels of adhesion molecules. Foam cells within the plaque aggravate inflammatory response mediated by the release of chemokines and growth factors and may undergo apoptosis (Tall and Yvan-Charvet, 2015).

As a result, the endothelium is activated by these stimuli and adhesion molecules such as vascular cell adhesion molecule-1 (*VCAM-1*) are upregulated in addition to T-cell and monocyte recruitment. Other activated adhesion molecules during this process are *P-selectin*, intercellular adhesion molecule-1 (*ICAM-1*) and *E-selectin*, that are capable of luring blood cells into the lesion. Transendothelial migration of blood-borne cells (monocytes and T-lymphocytes) to the atherosclerotic lesion also requires chemotactic cytokines (atherogenic chemoattractants) – chemokine ligand 2 (*CCL-2*) and oxLDL (Libby Peter, 2002). Monocyte derived macrophages within the intima are capable of recruiting themselves by secreting *CCL-2*. Many pro-atherogenic cytokines such as interleukin-1 (*IL-1*) and interleukin-6 (*IL-6*) are upregulated in atherosclerotic plaques.

Targeting the receptors of these cytokines could help hinder the inflammatory process associated with this disease and also remove the necrotic foam cells in the plaque.

Today, it is crucial for cardiovascular researchers to explore the inherent pathology of atherosclerosis in order to better understand the pathophysiology of this disease and eventually, identify potential therapeutic targets.

1.2 Cholesterol and Cholesterol Carriers

Cholesterol is synthesised by the liver and can also be ingested from food. The body cells require cholesterol for their functioning, for example, in cell membrane formation and it is also an essential material for the synthesis of steroid hormones, bile and vitamin. Cholesterol molecules are packaged and carried by lipoproteins. Lipoproteins are small singe-layer phospholipid membrane particles made up of fat, protein and cholesterol. Plasma lipoproteins include; high-density lipoprotein (HDL), low-density lipoprotein (LDL), intermediate-density lipoprotein (IDL), very low density lipoprotein (VLDL) and chylomicrons, which facilitate the movement of fat and cholesterol via the bloodstream. These cholesterol-carriers contain different proportion of triglycerides, cholesterol and also differ by density. The lower the density of the lipoprotein, the higher is the fat content within it. The level of cholesterol in the cells must be managed within a narrow limit. An excess or too little cellular cholesterol could disrupt the cell membrane and/or causes necrosis and apoptosis (Tabas Ira, 2002). Cells can obtain the required cholesterol from lipoproteins in the plasma and from intracellular synthesis thus, cholesterol deficiency in cells is a rare occurrence. However, excess systemic cholesterol is a common phenomenon. Since cells are not able to degrade cholesterol, with the exception of adrenocortical cells and hepatocytes, reduction of cellular cholesterol content is either via cholesterol efflux or conversion of cholesterol to cholesteryl esters. The latter though, can also be toxic when cells are overloaded with cholesterol, unlike cholesterol efflux (Low *et al*; 2012).

Increased blood cholesterol level puts an individual in high risk of heart and circulatory diseases. This has no obvious symptoms, but the individual has a marked risk of developing conditions such as atherosclerosis, heart attack, stroke and other CVDs.

A simple blood test (lipoprotein panel) could help identify people at high risk of developing coronary heart disease by measuring total body cholesterol, LDL, HDL and triglycerides levels (NIH Medline Plus, 2012). The table below (Table 1) shows the desirable levels of total cholesterol and its fractions.

Desirable Cholesterol Level		
Total Cholesterol	< 200 mg/dL	< 4.0 mmol/L
LDL	< 100 mg/dL	< 3.0 mmol/L
HDL	≥ 60 mg/dL	≥ 1.5 mmol/L
Triglycerides	< 150 mg/dL	< 3.0 mmol/L

Table 1 Desirable blood cholesterol level (Adapted from NIH Publication No. 01 – 3670 and HEART UK)

A raised and unhealthy pattern of cholesterol noticed in some individual generally depends on both controllable and uncontrollable factors. Excess consumption of diet enriched in saturated fat and cholesterol, in addition to being overweight, and idle lifestyle contribute to the controllable risk factors of heightened blood cholesterol level. Physical exercises and loosing excessive body weight help to lower the circulating levels of total cholesterol, LDL-C and triglycerides. In return, the level of HDL is raised. On the other hand, age, gender, heredity, medical history and ethnicity may predispose certain individuals to higher blood cholesterol. These elements are uncontrollable risk factors, which may subsequently contribute to the development of circulatory and heart diseases.

Also, impairment in the fat and cholesterol transportation may pose a major health consequence. Such malfunction could increase the concentration of LDL in blood and provoke atherogenesis. Ideally, the ratio of LDL to HDL should be low. There is a correlation between heart diseases and high level of LDL (“bad cholesterol”). The risk of developing a CVD increases when LDL particles become oxidised following the invasion of endothelial lining of blood vessels. Over time, the rate of atherosclerosis development increases on the arterial walls as they attract macrophages. The so-called “good cholesterol” (HDL) helps to alleviate the occurrence of atherosclerosis, if in higher concentration in the blood. HDL transportation of fat molecules away from artery walls prevents or reduces the progression of atherosclerosis.

While apolipoprotein A (ApoA) serve as the major protein component of HDL, apolipoprotein B (ApoB) is the primary apolipoprotein of chylomicrons, VLDL, IDL and LDL particles (Wasan *et al*; 2008). An important lipoprotein in the modelling of atherosclerosis in mice is the apolipoprotein E (ApoE). ApoE is found on the surface of most lipoprotein particles, which play a vital role in the clearance of triglycerides and cholesterol from the blood. It is mainly produced by the liver and macrophages and it is essential in the metabolism of cholesterol as well as transportation of lipoprotein, cholesterol and fat-soluble vitamins to the lymph system, then into the blood (Baars *et al*; 2011). The role of ApoE in atherosclerosis development has been ascertained in a gene knock-out mouse model, namely in $ApoE^{-/-}$ mice. It interacts with LDL receptor family and heparan sulfate proteoglycans to enhance lipoprotein metabolism. ApoE is an important molecule in the onset of atherosclerosis in the coronary arteries since it plays a role in reverse cholesterol transport (RCT) process, when it influence the ability of HDL particles in mediating efflux of lipid from cells (Davignon *et al*; 1988). Hypercholesterolaemia and extensive atherosclerotic lesions development are usually observed in $ApoE^{-/-}$ mice even when on a chow diet. These characteristics of ApoE knockout mice are more pronounced when put on a western (high fat) diet. This is a result of impaired clearance of chylomicrons and VLDL. Consequently, increased plasma triglycerides and cholesterol ensue in addition to the development of substantial amount of atherosclerotic lesions (Jawien J, 2012).

1.3 Cholesterol Efflux and Reverse Cholesterol Transporters

Cholesterol and triglycerides are important part of the body physiology. These are key components of cellular structure and function. Hypercholesterolaemia however, enhances cholesterol accumulation in monocyte-derived macrophages and other leukocytes, which in turn, promotes inflammatory responses such as augmentation of Toll-like receptor (TLR) signaling, activation of inflammasome and the secretion of neutrophils and monocytes in the spleen and bone marrow. The activation of TLR signaling results in decreased cholesterol efflux, consequently increasing cholesterol accumulation and escalates inflammatory responses (Tall and Yvan-Charvet, 2015).

On the arterial wall, the cholesterol contained in the cholesterol laden macrophages is the main inflammatory driver in atheroma build-up.

This cholesterol is eventually efflux from the macrophage primarily by ATP-binding cassette transporters such as *ABCA1* and *ABCG1*. These cholesterol transporters initiate the process of RCT via the enhancement of cholesterol efflux from macrophages to ApoA-I and HDL. Cholesterol transported to the liver are either recycled into lipoprotein or further excreted into bile through *ABCG5* and *ABCG8* transporters.

HDL is thought to be an anti-atherogenic lipoprotein since it is inversely linked with the risk of atherosclerosis development. The anti-atherogenic functions of HDL are due to its ability to intrinsically transport proteins, anti-oxidant capacity, anti-coagulative, anti-inflammatory and nitric oxide-promoting properties, as well as mediation of macrophage cholesterol efflux. HDL assist in the maintenance of net cholesterol concentration in the arterial wall as it readily accepts cholesterol from cholesterol-rich macrophages thereby, alleviating pro-inflammatory responses (Rosenson *et al*; 2011). HDL confers protection against the induction of atherosclerosis by promoting the efflux of cholesterol from macrophages via liver, bile and feces - a mechanism known as RCT. This process involves cholesterol transportation from peripheral tissues and macrophages via plasma HDL to the liver for ultimate fecal excretion (**Fig. 2**). The concentration of cholesterol in HDL is proportional to the efficiency of RCT process. A critical starting step of RCT involves the efflux of cholesterol from macrophages to plasma HDL particles. An important pathway for this process includes the interaction between cholesterol-deficient ApoA-I complexes and *ABCA1*. This is followed by *ABCG1* mediating the interaction of cholesterol efflux from macrophage with cholesterol-containing α-HDL particles (Timmins *et al*; 2005). On the other hand, the scavenger receptor class B type I (*SR-BI*) mediates bidirectional lipid transportation in the macrophage. And, this is dependent on the content of cholesterol in the lipid-containing macrophages. However, *SR-BI* is more prominent in cholesterol trafficking by the liver via selective uptake of cholesteryl esters from mature HDL.

In the liver, strolin-1 and strolin-2 proteins encoded by *ABCG5* and *ABCG8* respectively facilitate the biliary excretion of dietary sterol (cholesterol) (Peter *et al*; 2011).

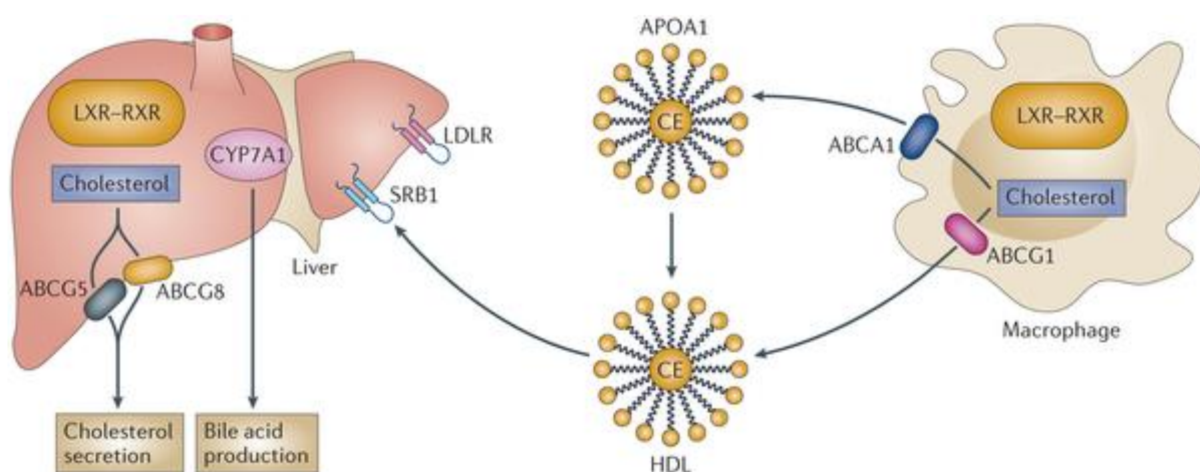

Fig. 2 Reverse cholesterol transportation (RCT) (Adapted from Cynthia Hong and Peter Tontonoz; 2014)

1.4 Melanocortins and the Melanocortin System

The melanocortin system is involved in diverse physiological functions including energy homeostasis, sexual function, pigmentation, exocrine function, pain relief, steroidogenesis, cardiovascular regulation, temperature regulation, immunomodulation, neuromuscular regeneration and inflammation (Catania *et al*; 2004). Genetics studies and knockout mouse models have further elucidated the role of melanocortins in inflammation regulation (Hetal *et al*; 2011). This family of peptides are derived from propopiomelanocortin (POMC) prohormone and are expressed in the central nervous system as well as in the peripheral tissues. They consist of adrenocorticotropic hormone (ACTH) and α-, β-, and γ-melanocyte-stimulating hormone (α-, β-, γ-MSH). Melanocortin peptides have affinity for five melanocortin receptors (MCRs), which are G-protein coupled and stimulate cAMP pathway through stimulatory G_s protein and adenylate cyclase (**Fig. 3**).

The affinities for MCRs by melanocortins differ. MCR activation is also associated with inositol trisphosphate (IP_3), MAP Kinase, extracellular Ca^{2+} influx, phosphokinase C (PKC) and Janus kinase pathway (Kapas *et al*; 1995). Melanocortin-1 (MC1-R) and melanocortin-3 (MC3-R) receptors are particularly involved in the inflammatory process.

MCRs regulate inflammation by hindering the activation of leukocytes to the atherogenic site in the artery. Macrophages express melanocortins and their receptors, namely MC1-R and MC3-R are functionally active on these cells (Hetal *et al;* 2011). Extensive experimental and clinical investigation on these MCR subtypes shows that their activation, especially that of MC1-R, has a strong potential for the management of inflammatory disorders (Catania *et al;* 2004, Rinne *et al;* 2015, Rinne *et al;* 2017). MC1-R is expressed by a variety of leukocytes such as monocytes and macrophages and by other cells involved in the pathophysiology of atherosclerosis. The activation of MC1-R inhibits the action of pro-inflammatory molecules such as *IL-1*, nitric oxide, *IL-6*, *TNF-α*, *CCL-2* and *INFγ* via the modulation of *NF-κB* transcription factor (Ira and Tung, 2003).

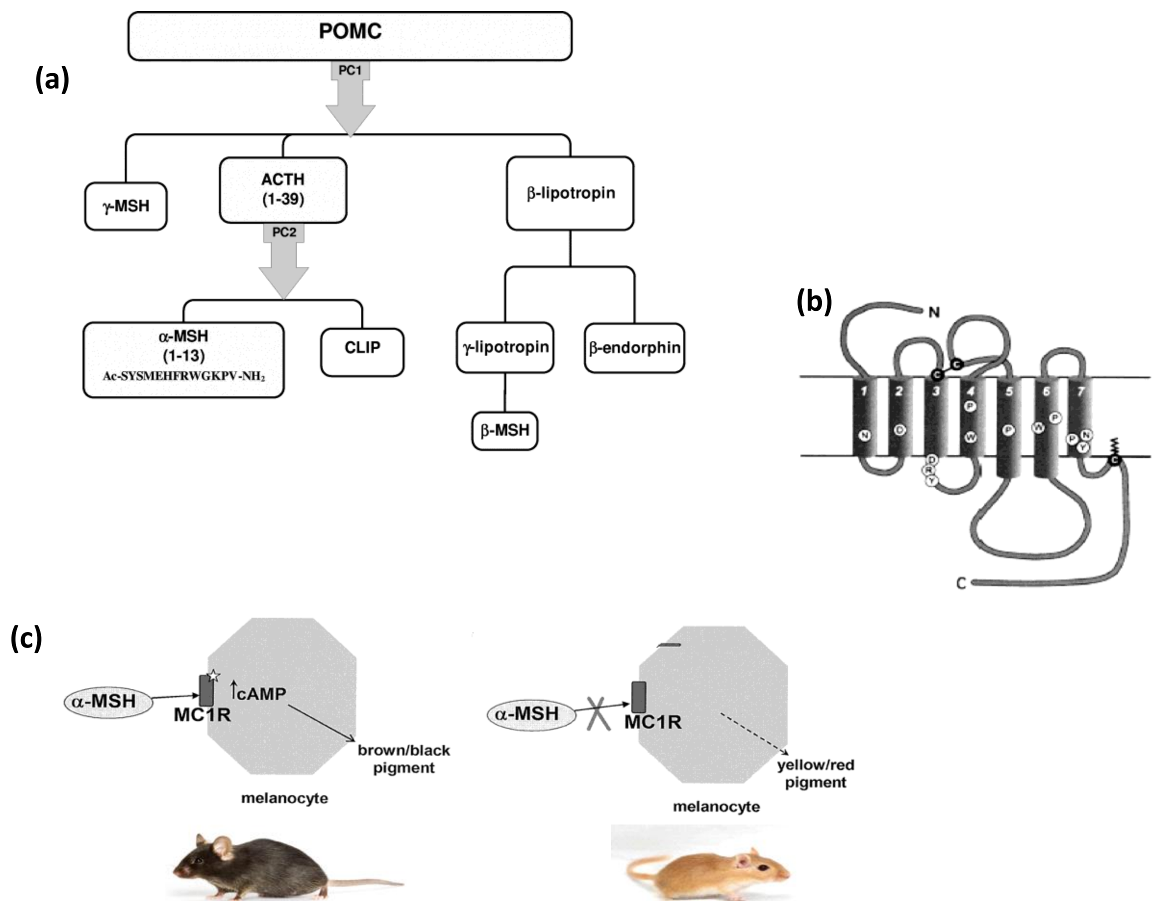

Fig. 3 (a) the melanocortin system (b) melanocortin 7-TM (GPCR) receptor (c) MC1-R activation & deficiency

α-MSH secreted by macrophages possesses anti-inflammatory properties via counteracting the effect of pro-inflammatory cytokines, nitric oxide and chemokines. MCR activation in macrophages inhibits leukocyte chemoattraction, induce expression of heme oxygenase-1 and increases phagocytosis and efferocytosis (Hetal *et al*; 2011). In a recent studies by Rinne *et al*, MC1-R deficiency was directly linked to increased arterial stiffness and impaired endothelial function (Rinne *et al*; 2015). Furthermore, a previous study by this group concluded, that the activation of melanocortin receptor is effective in the limitation of plaque inflammation and improved arterial function in atherosclerotic mice (Rinne *et al*; 2014).

Interestingly, a recent investigation revealed that MC1-R agonist activation in macrophages reduces oxLDL uptake via the down regulation of *CD36* cell surface expression and increases *ABCA1* and *ABCG1* expression, thereby promoting RCT (Rinne *et al*; 2017). The link between melanocortin biology and atherosclerosis development in mice is a relatively new area of the disease. In this Master's thesis, we will further investigate the relationship between MC1-R and cholesterol transporter genes in atherosclerotic mouse model. Specifically, we aim to study how MC1-R deficiency affects the expression of cholesterol transporter genes in atherosclerotic ApoE$^{-/-}$ mice. Furthermore, we will explore whether there is a functional consequence of MC1-R deficiency in terms of *in vivo* reverse cholesterol transportation. We hypothesise that MC1-R deficiency leads to decreased expression of reverse cholesterol transportation genes and consequently, impact the efflux of cholesterol from monocyte-derived macrophages and the process of RCT.

2. RESULTS

2.1 *ABCA1* and *ABCG1* Cholesterol Transporters are Down-regulated in the Aorta of MC1-R Deficient Mice

C57BL/6J mice of ApoE$^{-/-}$ background were divided into two experimental groups; chow diet fed mice and four weeks western diet group of mice. Each group of mice contained two genotypes subgroups; ApoE$^{-/-}$ wild type (WT) black mice and yellow ApoE$^{-/-}$ MC1-R$^{e/e}$ mice. Tissue samples (20mg - 50mg) with high relevance to the pathogenesis of atherosclerosis (aorta, liver, spleen and bone marrow) were obtained from these mice after sacrifice. RNA extraction was carried out with QIAGEN RNA kit and reverse transcribed to cDNA (Takara cDNA synthesis kit). Finally, the expression of cholesterol transporter genes such as *ABCA1, ABCG1,* and *CD36, SR-A1*; cytokines such as *IL-1β, IL-6, CCL-2*; stability genes *Col2A1, Col3A1, αSMA* etc; and other tissue-specific cholesterol transporters were quantified by quantitative PCR (qPCR). The expression of target protein was normalised to *S29 and β-actin* or hypoxanthine phosphoribosyltransferase (*HPRT*). ΔCt comparative method was used to calculate the fold induction and were presented as relative transcript levels ($2^{-\Delta\Delta Ct}$).

Table 2: Gene Expression in the Aorta of Mice

Gene	Chow		4 Weeks High Fat Diet (HFD)	
	WT	*MC1-R $^{e/e}$*	*WT*	*MC1-R $^{e/e}$*
ABCA1	1.06 ±0.15	0.69 ±0.14	1.86 ±0.18	1.47 ±0.19
ABCG1	1.23 ±0.36	0.56 ±0.05	2.33 ±0.24	1.51 ±0.28
CD36	1.44 ±0.62	1.76 ±0.75	3.82 ±0.76	2.55 ±0.60
SRA-1	1.27 ±0.45	0.87 ±0.13	1.29 ±0.14	4.65 ±3.68
SRB-1	1.09 ±0.19	1.00 ±0.32	2.28 ±0.87	1.24 ±0.25
IL-6	2.71 ±1.62	1.80 ±0.78	5.65 ±1.81	4.46 ±2.46
IL-1β	1.23 ±0.29	0.42 ±0.07	3.26 ±1.01	1.34 ±0.34
CCL-2	1.42 ±0.36	0.47 ±0.19	2.33 ±0.69	0.71 ±0.20
CCL-5	1.13 ±0.20	1.51 ±0.19	1.72 ±0.55	1.53 ±0.35
VCAM-1	1.03 ±0.09	0.89 ±0.16	1.28 ±0.25	1.41 ±0.20
αSMA	1.50 ±0.49	1.31 ±0.50	1.53 ±0.32	0.90 ±0.10

COL1A2	1.05 ±0.13	1.28 ±0.17	0.52 ±0.09	0.76 ±0.15
COL3A1	1.10 ±0.19	0.72 ±0.07	1.46 ±0.22	0.93 ±0.14
COL1A1	1.11 ±0.21	1.25 ±0.31	0.94 ±0.29	0.62 ±0.11
COL4A1	1.04 ±0.11	1.14 ±0.12	0.94 ±0.12	1.02 ±0.15
MMP2	1.58 ±0.65	1.09 ±0.45	2.08 ±0.56	2.62 ±0.98
TGFβ1	1.01 ±0.06	1.23 ±0.14	1.13 ±0.11	1.54 ±0.16
ARG1	0.80 ±0.67	0.73 ±0.50	0.35 ±0.18	0.34 ±0.16
CD206	1.05 ±0.11	1.01 ±0.11	0.99 ±0.21	1.12 ±0.37
CX3CR1	1.10 ±0.17	0.63 ±0.05	2.09 ±0.44	1.05 ±0.25
ICAM1	1.18 ±0.28	0.82 ±0.11	0.55 ±0.09	0.68 ±0.12
PECAM1	1.12 ±0.18	0.98 ±0.15	1.10 ±0.21	1.34 ±0.21
CD62P	1.10 ±0.20	0.76 ±0.10	2.14 ±0.35	1.27 ±0.20

Fold change of genes expression (Mean ±S.E.M) in aorta of ApoE KO WT and MC1R$^{e/e}$ mice against the housekeeping genes (Geo.mean *S29* & *HPRT*).

In the aorta, the *ABCA1* and *ABCG1* expression conspicuously differ in the genotypes as well as between the diet groups (**Fig.4a & b**). MC1-R$^{e/e}$ mice show reduced amount of *ABCA1* and *ABCG1* expression in relation to the ApoE$^{-/-}$ wild type. These cholesterol carriers are chiefly expressed by macrophages and play a crucial role in cholesterol homeostasis, translocating cholesterol out of macrophages to HDL and ApoA-I. *ABCA1* and *ABCG1* reduced expression in MC1-R$^{e/e}$ mice may influence cholesterol efflux in this group of mice and impact *ABCA1*- and *ABCG1*-mediated RCT. On the other hand, expression of *CD36* did not indicate a significant difference between the genotypes (**Fig.4c**). The scavenger receptors facilitate the influx of oxLDL when expressed on the surface of macrophages.

Furthermore, collagenase type 3A1 and α-smooth muscle actin (*αSMA*) mRNA expression were down regulated in the aorta of MC1-R$^{e/e}$ mice (**Fig. 4d & e)**. These genes play a vital role in the structure and stability of the aortic wall. The characteristics of plaque formed in MC1-R deficient mice may thus be less stable to the constant flow of blood at higher pressure within the artery.

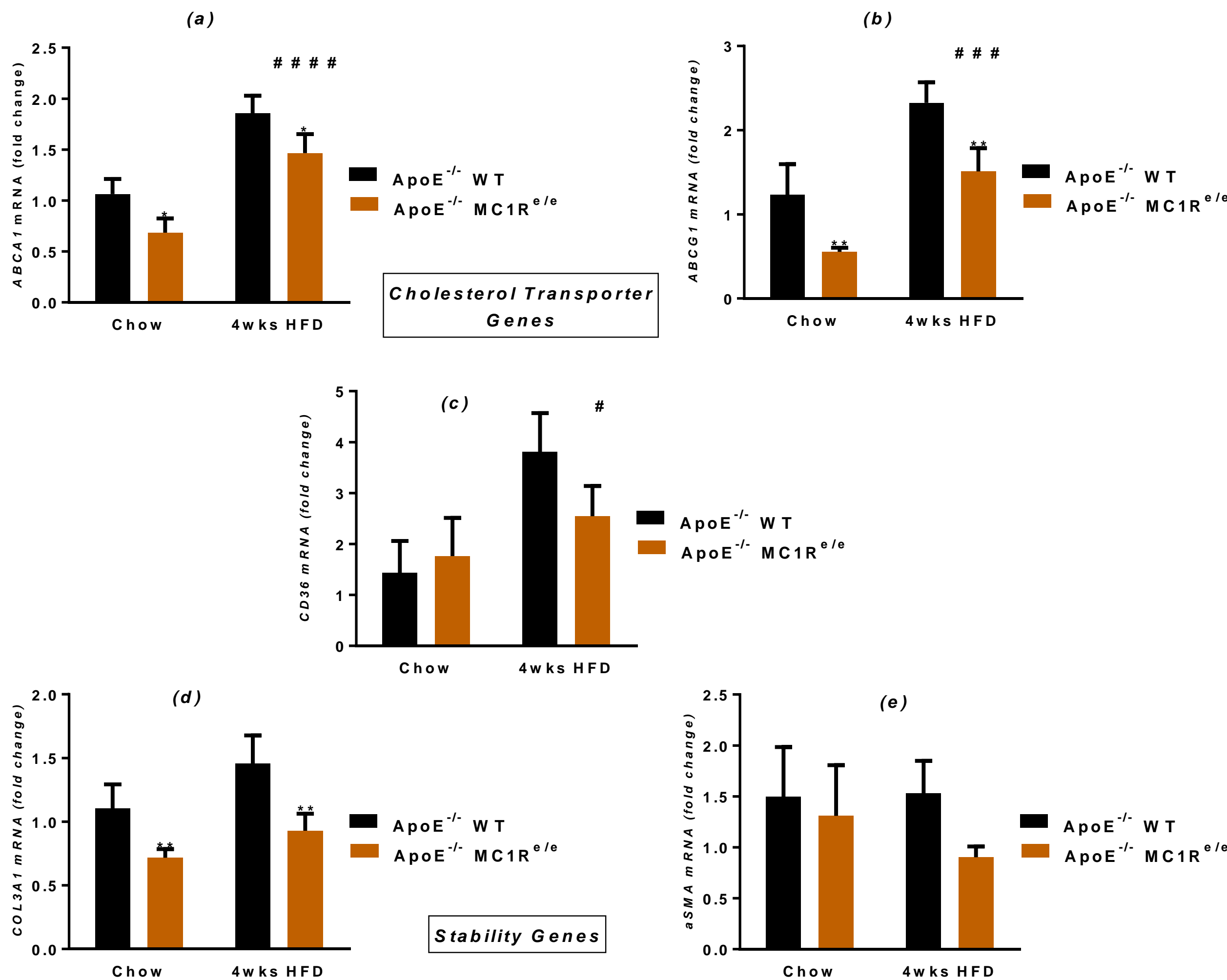

Fig 4: Cholesterol Transporter and Stability Genes mRNA Expression in the Aorta

Cholesterol transporter and stability genes mRNA expression in the aorta of WT and MC1R$^{e/e}$ mice fed with chow and 4wks HFD. (a) ABCA1 expression in aorta of chow and HFD mice. (b) ABCG1 expression level in aorta of the mice (c) Expression of CD36 mRNA in chow and HFD mice. (d) Col3A1 expression in chow and HFD mice. (e) aSMA mRNA expression.

*Gene expression was measured with qPCR and the data (fold change) is a quantity of the expressed mRNA.. (*P < 0.05, **P< 0.01 against WT); (###P < 0.001, ####P < 0.0001 = dietary significance); n=8 per group.*

The expression of cytokines, chemokines and adhesion molecules in the aorta of the mice was reduced in both diet groups and in MC1-R deficient genotype relatively to their ApoE$^{-/-}$ WT. Specifically, *IL-1β, CCL-2, IL-6* and *CD62P* genes were down-regulated in MC1-R deficient mice (**Fig.5a, b, c & d**). However, an upregulation of *TGB-1* mRNA was observed.

On the other hand, cytokines, chemokines and stability genes such as *CD206, CCL-2, CCL-5, VCAM-1, ICAM1, PECAM1, Col1A1, Col1A2, Col1A4, MMP2* and *ARG1* expression in the aorta did not show significant difference between the two genotypes (**Table 2**).

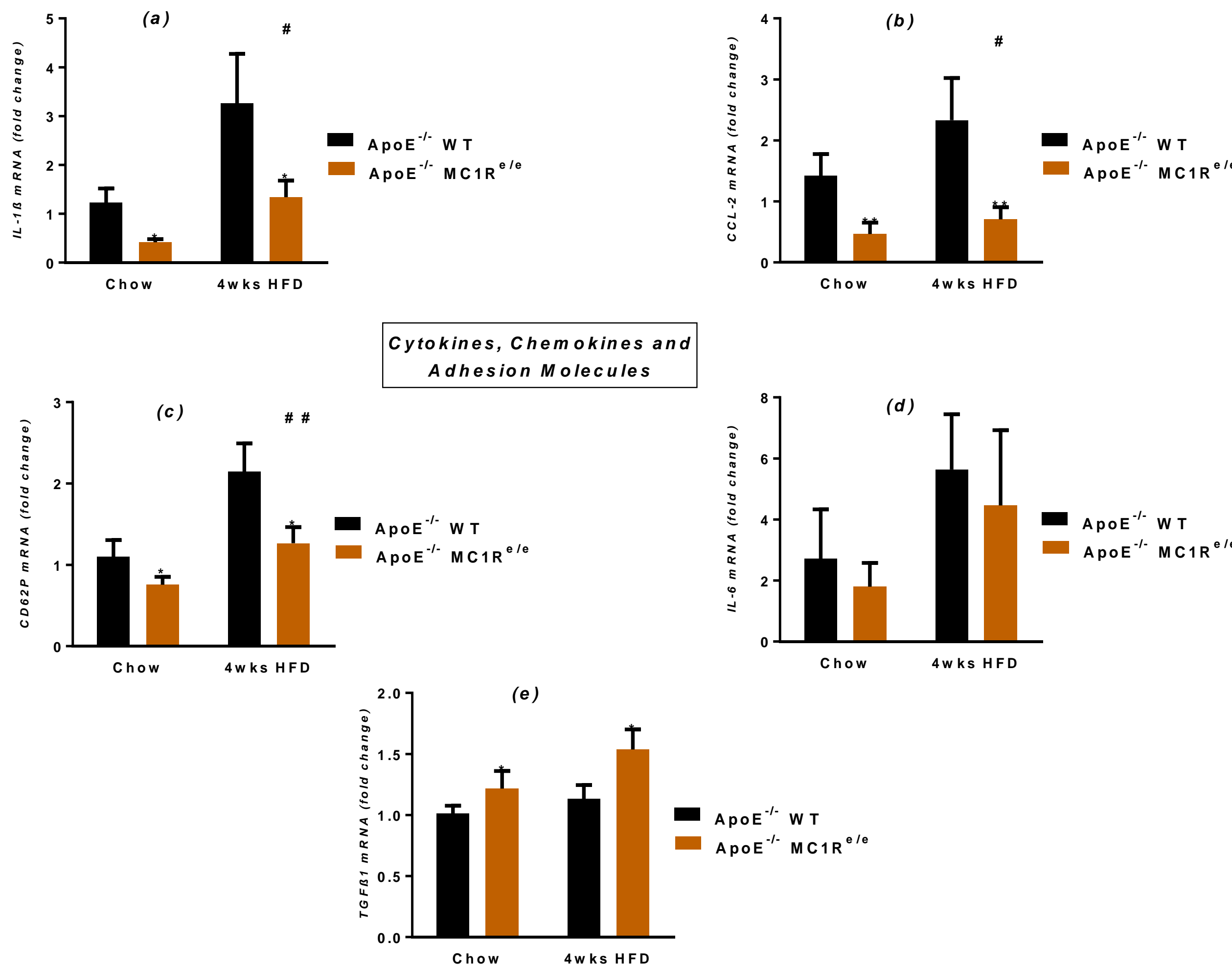

Fig. 5: Cytokines, Adhesion Molecules and Chemokines Gene Expression in Aorta

*Cytokines, adhesion molecules and chemokines gene expression in aorta of chow and 4wks HFD mice. (a) IL-1β mRNA expression level in aorta of chow and HFD mice (b) CCL-2 expressed in aorta of chow and 4weeks HFD mice (c) CD62P level detected in the aorta of chow and HFD mice (d) IL-6 quantification in aorta of chow and 4 weeks HFD fed mice. (e)TGFβ-1 expression in aorta of chow and HFD fed mice. (*P < 0.05, **P< 0.01 against WT); (#P < 0.05, ##P < 0.01 = dietary significance); n=8 per group.*

2.2 Deficiency in MC1-R Resulted in Reduced *ABCG5* and *ABCG8* Expression in the Liver of HFD-fed Mice

ABCG5 and *ABCG8* cholesterol transporters are the primary carriers of cholesterol delivered to the liver for bio-degradation in the bile. These cholesterol carriers were downregulated in MC1-$R^{e/e}$ mice, as the animals' system became over loaded with cholesterol from the 4 weeks western diet (**Fig.6a & b)**. In the chow diet fed mice, the liver expression of *ABCG5* and *ABCG8* transporter genes were somewhat equal in both genotypes (**Fig 6a & b**). The *SR-B1* scavenger receptors expressed on the surface of the liver, which facilitates the intake of cholesterol delivered by HDL did not show major difference between the genotypes or diet groups (**Table 3**).

Table 3: Gene Expression of Cholesterol Transporter Genes in the Liver of Mice

Gene	Chow		High Fat Diet (HFD)	
	WT	*MC1-R* $^{e/e}$	*WT*	*MC1-R* $^{e/e}$
ABCA1	1.02 ±0.05	0.97 ±0.04	0.95 ±0.04	0.94 ±0.08
ABCG1	1.01 ±0.06	1.34 ±0.12	2.27 ±0.20	3.21 ±0.50
CD36	1.11 ±0.21	1.56 ±0.33	5.43 ±0.69	6.42 ±0.96
SRA-1	1.03 ±0.10	1.24 ±0.06	1.93 ±0.15	2.13 ±0.26
SRB-1	1.01 ±0.04	0.99 ±0.07	1.17 ±0.11	1.27 ±0.09
ABCG5	1.08 ±0.16	1.88 ±0.12	3.80 ±0.32	2.71 ±0.26
ABCG8	1.07 ±0.15	1.45 ±0.09	3.19 ±0.26	2.33 ±0.19
LDL-r	1.01 ±0.06	1.00 ±0.11	0.44 ±0.05	0.58 ±0.10
LXRa	1.13 ±0.22	0.99 ±0.09	1.31 ±0.23	1.68 ±0.25
Apo-A1	1.06 ±0.14	1.15 ±0.06	1.10 ±0.15	0.97 ±0.10
SREBP	1.01 ±0.05	1.07 ±0.07	0.36 ±0.04	0.41 ±0.08

Fold change of different genes expression (Mean and S.E.M) in the liver of ApoE KO WT and MC1$R^{e/e}$ mice against the housekeeping genes (Geo.mean *S29* & *β-actin*).

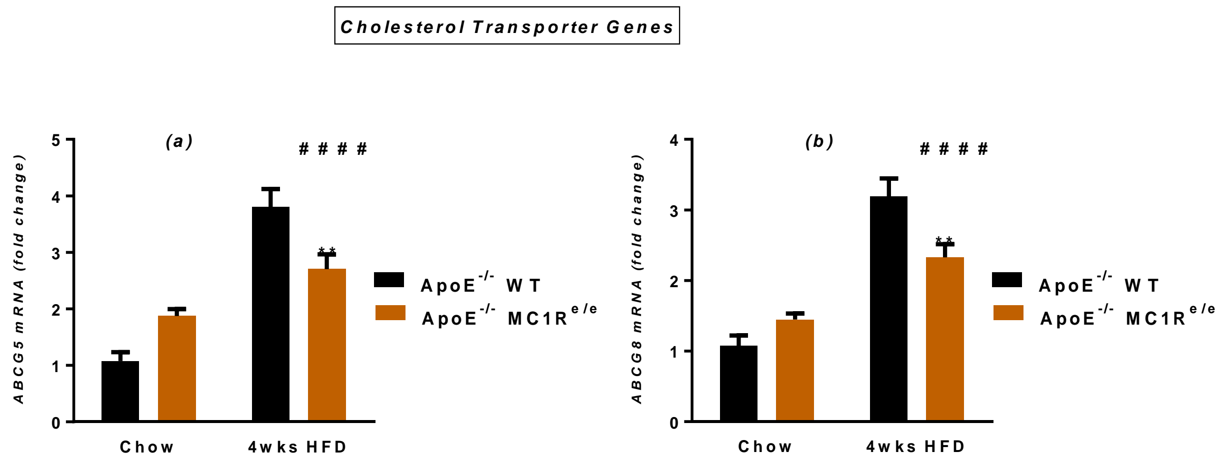

Fig. 6: Cholesterol Transporter Genes Expression Level in the Liver

Cholesterol transporter genes expression level in the liver of chow and 4wks HFD mice.
(a) ABCG5 and (b) ABCG8 expression in liver samples of both genotypes and diet groups of mice.
*(**P < 0.01 against WT); (####P < 0.0001 = dietary significance); n=8 per group.*

2.3 Cholesterol-Rich Diet Influence the Expression of Cholesterol Transporter Genes and Pro-inflammatory Mediators in the Spleen and Bone Marrow of Mice

In the spleen and bone marrow of mice, the expression of cholesterol transporter genes was unaffected by the genotype. However, the diet type altered the level of cholesterol transporter genes expression in these tissues (**Supplemental- Fig 1 & 2; Table 4 & 5**). Western diet fed mice showed an increase in cholesterol transporter gene expression in the spleen and bone marrow of both wild type and MC1-R$^{e/e}$ mice. The bone marrow and splenic macrophages responded to the increased overload of cholesterol in the circulatory system via successive increase in the *ABCA1* in bone marrow and *ABCG1* in spleen.

Table 4: Gene Expression in the Spleen of Mice

Gene	Chow		High Fat Diet (HFD)	
	WT	*MC1-R* $^{e/e}$	*WT*	*MC1-R* $^{e/e}$
ABCA1	1.01 ±0.05	1.08 ±0.07	1.10 ±0.15	1.24 ±0.12
ABCG1	1.01 ±0.06	0.90 ±0.04	1.31 ±0.18	1.33 ±0.10
CD36	1.04 ±0.11	1.22 ±0.16	0.57 ±0.06	0.79 ±0.07
SRB-1	1.01 ±0.07	1.09 ±0.13	0.77 ±0.05	0.86 ±0.09
IL-1β	1.05 ±0.12	0.82 ±0.11	0.76 ±0.15	0.56 ±0.09
CCL-2	1.10 ±0.18	1.65 ±0.28	0.60 ±0.15	0.48 ±0.14
VCAM-1	1.04 ±0.11	1.07 ±0.15	0.91 ±0.13	1.10 ±0.19

Fold change of different genes expression (Mean and S.E.M) in the spleen of ApoE KO WT and MC1R$^{e/e}$ mice against the *S29* housekeeping gene.

The cholesterol transporters (*ABCA1, ABCG1 and CD36*) and cytokine (*IL1β*, *CCL-2*) mRNA expression levels in the spleen of both groups of mice were expressed differently between the genotypes, but with no statistical difference (**Supplemental Fig. 1 & 2**). However, the diet type seems to play a critical role in the expression level of these cholesterol transporter and cytokine genes that are being expressed (**Supplemental - Fig.1a, b, c & d**). In a similar pattern as in the spleen, the bone marrow expression of cholesterol transporter genes and scavenger receptors did not show any significant differences between the genotypes (**Supplemental - Fig.2a, b, c & d**). This outcome also translates similarly to the expression of *IL1β* and *CCL-2* in the spleen, where the observable differences are seen in the diet and not in the genotype (**Supplemental Fig.1c & d**).

Table 5: Gene Expression of Cholesterol Transporters Genes in the Bone Marrow of Mice

Gene	Chow		High Fat Diet (HFD)	
	WT	*MC1-R* $^{e/e}$	*WT*	*MC1-R* $^{e/e}$
ABCA1	1.04 ±0.12	1.33 ±0.14	5.12 ±0.42	4.00 ±0.67
ABCG1	1.03 ±0.09	1.14 ±0.13	1.64 ±0.27	1.12 ±0.06
CD36	1.15 ±0.24	1.66 ±0.43	3.65 ±0.31	3.93 ±0.67
SRA-1	1.06 ±0.13	1.36 ±0.17	1.09 ±0.17	0.96 ±0.17
SRB-1	1.02 ±0.08	0.90 ±0.06	1.52 ±0.09	1.32 ±0.16

Fold change of different genes expression (Mean and S.E.M) in the bone marrow of ApoE KO WT and MC1R$^{e/e}$ mice against the housekeeping genes (Geo.mean *S29* & *β-actin*).

2.4 H^3-Cholesterol Absorption into Plasma, Liver and Excretion in Feces of Mice via Cholesterol Efflux Mechanism

The *in-vivo* cholesterol efflux assay investigation was designed to expatiate on the level and distribution of free cholesterol in tissues such as liver, plasma and feces in both genotypes. The aim of this experiment was to measure the efficiency of cholesterol efflux from H^3-cholesterol loaded macrophages between the genotypes. Differentiated bone marrow-derived macrophages (BMDM) from normal ApoE$^{-/-}$ WT mice were loaded with H^3-cholesterol and injected intraperitoneally into the ApoE$^{-/-}$ WT and ApoE$^{-/-}$ MC1-R$^{e/e}$ yellow mice. After 24 to 48hours, tritiated cholesterol level in the collected plasma, liver and feces was measured in a liquid scintillation counter. The mice used in this experiment were on normal chow diet.

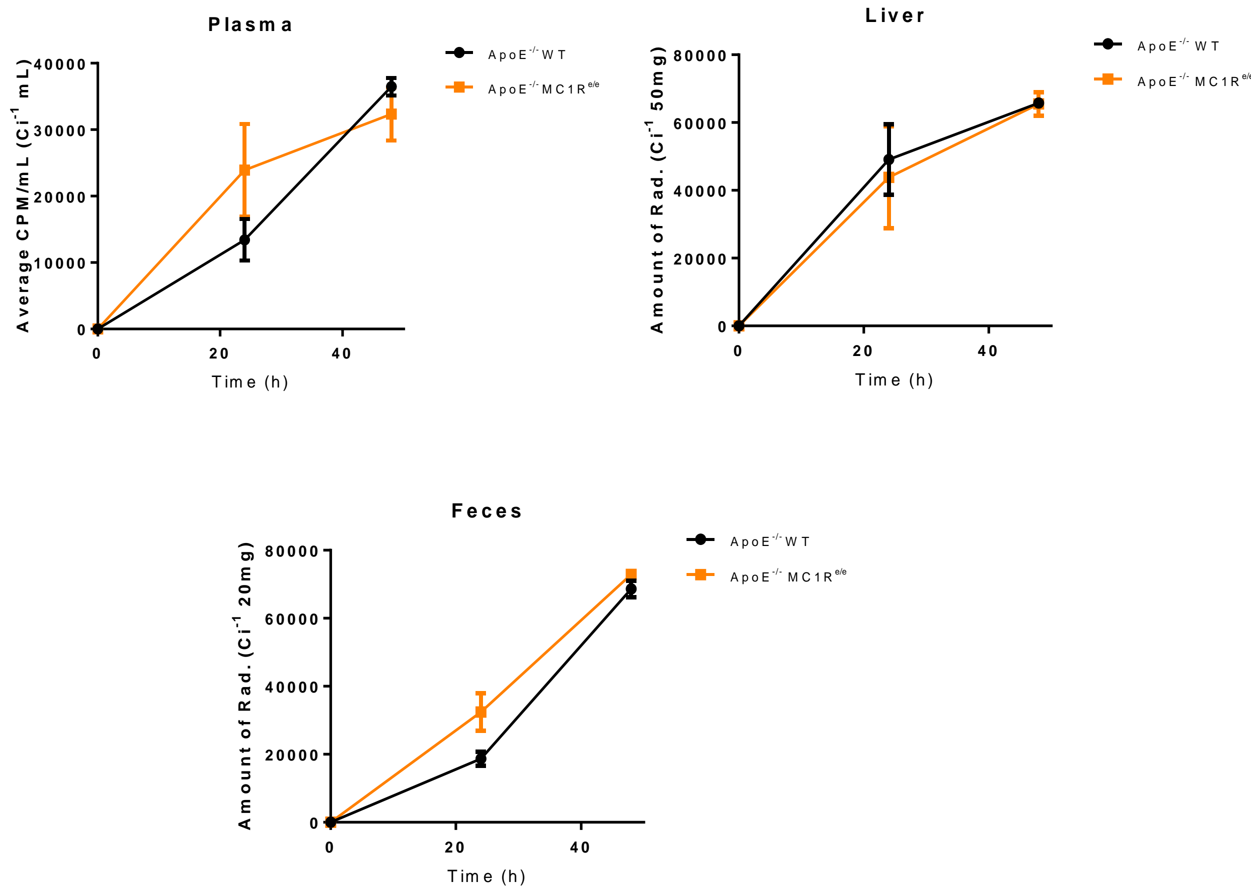

Fig. 7: H^3-Cholesterol Quantification in Plasma, Liver and Feces of Mice

Amount of H^3-cholesterol in liver, feces and plasma of WT and MC1R deficient mice after 24 to 48hrs of macrophage ip injection.

The graphs show that the tritiated cholesterol was absorbed in the blood, transported to the liver and eventually expelled via feces after 24 and 48 hours. However, there was no major difference between the genotypes in this regard.

3. DISCUSSION

This study was aimed at examining the effects of MC1-R deficiency on cholesterol transporter genes in mice that are prone to developing atherosclerosis, namely the $ApoE^{-/-}$ mice. We investigated the expression of these genes, in addition to inflammatory and stability genes in yellow recessive MC1-R deficient ($MC1\text{-}R^{e/e}$) and their littermate black wild type mice of $ApoE^{-/-}$ background. Both groups of mice had dietary sub-groups of chow and high fat diet (4 weeks HFD).

We observed in this study, that important cholesterol carrier genes – *ABCA1* and *ABCG1* were less expressed in the aorta of $MC1\text{-}R^{e/e}$ mice relatively to the wild type mice (**Fig. 4a & b**). A similar event was noted in the levels of *ABCG5* and *ABCG8* transporter genes in the liver, when mice were exposed to HFD for 4 weeks. Thus, we can conclude that MC1-R has an impact on the expression of cholesterol transporter genes, which in turn, may affect the transportation of cholesterol within the tissues and consequently, contribute to the inflammatory process preceding atheroma buildup in aorta. The role of *ABCA1* and *ABCG1* is well documented in the initiation of reverse cholesterol transportation. A recent study by Rinne *et al* supported this finding, when MC1-R was activated by MC1-R agonists in $ApoE^{-/-}$ mice (Rinne *et al;* 2017). The macrophages were hindered from excess cholesterol accumulation and foam cell development following the administration of MC1-R agents. The decrease expression of *ABCA1* and *ABCG1* mRNA in the aorta of chow and high fat diet mice (**Fig. 4a & b**) was likely due to lower biological effect of these genes, when MC1-R expression is deficient. Hence, cholesterol efflux, a counter regulatory mechanism against the formation of foam cell is in turn affected. Another important contributing factor to foam cell development in the aorta is the expression of scavenger receptor *CD36*. Although in this experiment, the expression of *CD36* mRNA (**Fig.4c**) in $MC1\text{-}R^{e/e}$ mice was not significantly changed relatively to the $ApoE^{-/-}$ wild type, previous experiment indicates that *CD36* was down-regulated when MC1-R was activated (Rinne *et al*; 2017). Interestingly, we saw a slight increase in *CD36* expression in $MC1\text{-}R^{e/e}$ mice in the chow diet fed mice (**Fig.4c**), but this did not reflect in the 4 weeks HFD fed mice.

ABCA1 and *ABCG1* transportation of cholesterol to HDL and ApoA-I in the aorta of the MC1-R$^{e/e}$ mice may have been impaired based on our results from this experiment. The spontaneous deletion of MC1-R in these yellow recessive mice may have affected the function of the cholesterol transporters (*ABCA1* and *ABCG1*) in transporting cholesterol laden LDL from the plaque in the arterial wall to the liver. This outcome second the conclusion of a recent investigation, which concluded that the activation of MC1-R agonist in macrophages leads to reduction in oxLDL uptake via down regulation of *CD36* cell surface expression and promote the process of reverse cholesterol transportation by increasing *ABCA1* and *ABCG1* expression (Rinne *et al;* 2017). The biological mechanism between MC1-R deficiency and the down-regulation of cholesterol transporter genes is not fully understood yet.

The activation of MC1-R by endogenous α-MSH through cAMP dependent pathway results in the inhibition of pro-inflammatory molecules such as *IL- 1β, IL-6, NO, TNF-α* and *INFγ* via *NF-κB* transcription factor and activation of PKA (Horrell *et al;* 2016). Hypercholesterolaemia allows cholesterol to build up in macrophages, the central cells in atherosclerosis disease derived from monocytes in circulating blood and other immune cells. This is followed by a cascade of inflammatory events that is regulated by the induction of different cytokines including interleukins, chemokines and adhesion molecules. The expression levels of *IL-1β*, *CCL-2* and P-selectin (*CD62P*) in the aorta of MC1-R deficient mice were significantly reduced. Also, *IL-6* mRNA expression level followed a similar pattern as *IL-1β*, *CCL-2* and *CD62P* except the lack of significance in expression between the genotypes (**Fig 5**). The down-regulation of these inflammatory players in the aorta was an unexpected finding, since hypercholesterolaemia, a pro-inflammatory event, generally increases the induction of inflammatory molecules (Stoke *et al*; 2002). This manifestation corresponds to an observation from a previous experiment, where it was observed that MC1-R$^{e/e}$ macrophages expressed lower levels of cytokines mRNA but still secreted a comparable amount of cytokine proteins (Rinne *et al;* 2017)**.** The reason(s) behind this is not completely clear yet. However, we speculate that the lower expression level of cytokines mRNA by MC1-R deficient mice was stable and biologically active.

Melanocortin receptor subtypes other than MC1-R also have a role in anti-inflammatory regulation in murine macrophages, which could explain the reduction of cytokine mRNA expression in MC1-R$^{e/e}$ mice. In **Fig 5**(**e**), *TGF-β1* expression was upregulated in the MC1R$^{e/e}$ mice on chow and western diets. *TGF-β1* cytokine/growth factor has been linked to the pathogenesis of atherosclerosis. The inhibition of *TGFβ1* signaling tends to inflate atherosclerosis progression and induce plaque instability in mice. Mallat *et al* show in their studies that the inhibition of *TGF-β1* signaling in ApoE$^{-/-}$ mice promoted atherosclerotic lesion development as well as increased inflammatory molecules and decreased collagen content. This finding suggests a protective role by *TGF-β1* in atherosclerosis. *TGF-β1* acts in cell cycle regulation and apoptosis in different types of cells including smooth muscle and endothelial cells. It also functions as an anti-inflammatory cytokine by attenuating the cytokine-regulated release of chemokines and adhesion molecules (Vodovotz *et al;* 1993). The atheroprotective role of *TGF-β* is linked to its deactivating effects on macrophages and T-lymphocytes and is unrelated to its cell differentiation effects on smooth muscle cells (Mallat *et al;* 2001). We observed in this experiment (**Fig 5e**), an upregulation of *TGF-β1* mRNA in MC1-R$^{e/e}$ mice in both diet groups. The possible physiological reason of this outcome is not well understood. However, *TGF-β* has anti-inflammatory effects via the reduction of cytokine-induced expression of chemokines and adhesion molecules.

ABCG5 and *ABCG8* promote the excretion of cholesterol into the gut lumen and bile, since they are exclusively expressed in the intestines and liver (Berge *et al*; 2000). These cholesterol transporters are co-expressed to traffic cholesterol from the endoplasmic reticulum site of synthesis in hepatocytes. Although the molecular mechanism of *ABCG5/G8* cholesterol translocation is not clear yet, the heterodimers show ATPase activity and interact with sterols probably using this energy to translocate the sterol across the membrane (Muller *et al;* 2006). In mice, the absence of these two transporter genes resulted in reduced cholesterol excretion into bile, while in transgenic mice, an enhanced biliary secretion of cholesterol was observed as well as a reduction in the amount of dietary sterol absorbed (Yu *et al;* 2002). In an *in vitro* condition, Vrins and co-workers (Vrins *et al* 2007) show that cells expressing *ABCG5* and *ABCG8* transporters promote the efflux of cholesterol into bile acids.

ABCG5 and *ABCG8* cholesterol transporter gene expression was reduced in the liver of MC1-$R^{e/e}$ mice relative to the $ApoE^{-/-}$ wild type after 4 weeks on western diet **(Fig 6a and b).** The down-regulation of *ABCG5* and *ABCG8* was not observed with chow diet condition. Mice on normal diet express similar level of mRNA for both *ABCG5* and *ABCG8* transporters. The level of cholesterol in the system probably mediates the level of expression of *ABCG5* and *ABCG8*. This finding suggests a probable link in the physiological function between MC1-R and *ABCG5* and *ABCG8* cholesterol transporter genes. In a previous study on the roles of *ABCG5* and *ABCG8* role in biliary cholesterol secretion, biliary cholesterol was substantially lower in $G5G8^{-/-}$ mice compare to wild type mice and only increased modestly when cholesterol-rich diet was given (Yu *et al;* 2002). This result stresses the important role of these two cholesterol transporter genes in hepatobiliary cholesterol transportation. In addition, *ABCG5* and *ABCG8* expression was found to prevent against sterol accumulation in the liver and intestine of mice as well as to mediate extrahepatic cholesterol efflux (Jin *et al;* 2015).

An overload of systemic cholesterol can result in the accumulation of cholesterol as well as macrophage mediated inflammation. Cholesterol efflux assay helps to quantify the rate at which macrophages efflux cholesterol. The cells ability to maintain this efflux as well as the capacity of plasma to accept the cholesterol being released from the cells can be measured by this assay. Essentially, cholesterol efflux assay demonstrates how a treatment of genetic manipulation affects cholesterol efflux capacity and how the plasma capacity to accept cholesterol is altered by a treatment or disease. **Figure 7** (cholesterol efflux assay) shows that the tritium labelled cholesterol loaded into macrophages was successfully accepted into the plasma of both wildtype and MC1-$R^{e/e}$ mice and transported into the liver. The radioactive H^3-cholesterol was transported to the liver, where it was processed and excreted via feces. MC1-$R^{e/e}$ mice appeared to excrete slightly more radioactive cholesterol after 24 hours of H^3-cholesterol injection, but this has no statistical significant between the genotypes.

This finding could explain the lack of difference in tritiated cholesterol efflux was as a result of no difference in expression level of *ABCG5* and *ABCG8* in the liver of chow-fed mice (**Fig 6a & b**).

There was no difference in the level of tritiated cholesterol. This may reflect efflux between the tissues or plsma (**Figure 7**). However, if these mice were fed with western diet, the outcome may have been different due to increase dietary cholesterol in the plasma. Further experiment vis-à-vis cholesterol efflux using HFD mice will be required to analyse any bearing that diet type may have in this assay.

The ApoE$^{-/-}$ mice model used in this investigation represent a widely used method in atherosclerosis research. This model allows the spontaneous development of atherosclerotic lesions even when mice are fed normal chow diet. In addition to this, feeding the mice with HFD also ensures a rapid buildup of cholesterol in the plasma. Perhaps, the addition of an 8 and/or 12 weeks HFD fed group of mice could have shown more pathological manifestation in the tissues analysed in this experiment and may also have impacted the expression level of cholesterol transporter genes. Atherosclerosis is a serious risk factor to CVDs. A number of treatments for CVDs are available today including surgery (e.g. coronary artery bypass). However, a non-invasive pharmacological therapy will be a preferred choice for the treatment of early atherosclerosis. The treatment of CVDs has had a positive breakthrough with the discovery of statins, which reduce blood cholesterol levels. However, other associative conditions and the underlying pathology still lack a concrete treatment, creating a demand for further research interest.

In conclusion, this investigation reveals that cholesterol transporter genes were downregulated in MC1-R$^{e/e}$ mice aorta and liver, an effect exacerbated with diet rich in high fat. The macrophages contained in the plaques of the MC1-R deficient mice may have reduced capability of cholesterol efflux hence hampering the process of RCT. The plaques formed in the aorta of this group may also be more vulnerable and thus, unstable as a result of reduced expression of *Col3A1* and *αSMA* mRNA. The outcomes of this investigation couple with results from previous studies by our group showing that MC1-R activation beneficially modifies the phenotype of atherosclerotic plaques.

This investigation was part of a bigger project and will be followed by a complementary study on plaque phenotyping, which will involve analyzing the impact of MC1-R deficiency on plaque size. A further study on the role of MC1-R on cholesterol homeostasis and trafficking in bigger mammals could proof a potential pharmacological therapy to ameliorate the advancement of atheroma in human atherosclerosis.

4. MATERIALS AND METHODS

4.1 Animals and Tissue Extraction

$ApoE^{-/-}$ C57B/J6 (black) and yellow $MC1\text{-}R^{e/e}$ were cross-bred to generate $ApoE^{-/-}$ and $ApoE^{-/-}$ $MC1\text{-}R^{e/e}$. CO_2 asphyxiation was used to sacrifice the mice. After euthanasia, samples from the aorta, liver, spleen and bone marrow were extracted and stored at -70 ^{o}C before further processing.

During this study, the welfare, health and quality of life of the mice were ensured. Mice had free access to the appropriate diet. All animal experiments were approved by the local Ethics committees (Animal Experiment Board in Finland, License Number: ESAVI-438 /04.10.03/2012) and conducted in accordance with the institutional and national guidelines for the care and use of laboratory animals.

4.2 RNA Extraction

Qiazol reagent (500 µL per 50 mg of tissue) was added to tissue in 2 mL eppendorf tubes and homogenised. 100 µL chloroform per 500 µL of Qiazol reagent was added and incubated at room temperature (RT) for 5 min. Samples were centrifuged at 12 000 x g (rcf) for 15 min at +4 ^{o}C and the clear upper phase was carefully pipetted into a 1.5 mL eppendorf tube. Then 250 µL of isopropanol (per 500 µL Qiazol used) was added to the mixture, vortexed and incubated at RT for 10 min. Samples were centrifuged at 12 000 x g (rcf) for 10 min at +4 ^{o}C. The supernatant was removed and 500 µL of 75% ethanol was added to the pellet, vortexed and centrifuged at 7500 x g for 5 min at +4 ^{o}C.

The supernatant was decanted and the pellet was allowed to air-dry for about 10 min in RT after which it was dissolved in RNAse free water for RNA concentration measurement with BioSpec-nano Spectrophotometer. Samples were stored at -70 ^{o}C until cDNA conversion.

4.3 cDNA Conversion

cDNA reverse transcription of the RNA samples was carried out with cDNA TAKARA PrimeScript RT Kit. The RNA sample was pipetted into small PCR eppendorf tubes and the master mix was added to the mixture. Samples were slightly centrifuged to get mixture at the bottom of the tube before setting in a thermal cycler (Applied Bioscience 2720) with the setting below:

	Step 1	Step 2	Step 3
Temperature (°C)	37	95	4
Time (min)	60	5	∞

The resultant cDNA was stored at -20 °C after the DNA amplification was completed.

4.4 Quantitative RT-PCR

DNA master mix for RT-PCR was prepared for each specific gene (housekeeping, cholesterol transporter, cytokines or stability genes) to run from their respective reverse and forward primers (Ambion), nuclease free water and SYBR green dye on ice. *S29* and/or *β-actin* housekeeping genes were readily expressed in mice' liver, spleen and bone marrow, while *S29* and *HPRT* expressed and stable in mice aorta. 3 µL of the cDNA sample (3 ng/µL) was pipetted in duplicate into a 96 PCR well plate on ice. 17 µL of DNA mix was added into each well and the plate was sealed with an adhesive film before setting the plate into a RT-PCR machine and with the following settings; enzyme activation 95 °C, 3:00min; denature 95 °C, 0:03min; anneal 60 °C, 0:27min. Dissociation stage was added to the setting and ran to obtain Ct-value readings in an Applied Bioscience – 7300 Real Time PCR System. The Ct-values obtained from the qPCR analysis is a relative quantification of expression of the target gene(s).

Target gene mRNA expression levels were analysed with Ms Excel package and normalized to the geometrical mean of ribosomal protein *S29* and *β–actin or HPRT* using the comparative ΔCt method and are presented as relative transcript levels ($2^{-\Delta\Delta Ct}$).

4.5 In-vivo Cholesterol Efflux Assay:

4.5.1 Macrophage Cell Culture and ^{3}H-Cholesterol Labelling

Step 1 (In-vitro Phase): Bone marrow cells were isolated from the femurs and tibiae of ApoE$^{-/-}$ mice and differentiated macrophages in M-CSF supplemented IMDM-high medium for 7 days. The cells were thereafter loaded with ^{3}H-cholesterol (5 µCi/mL) and AcLDL (30 µg/mL) in complete medium for 24 hours. Cells were washed twice with pre-warmed PBS and equilibrated with IMDM-high glucose medium containing 0.2% BSA for 4 hours.

The cells were further washed with pre-warmed PBS twice before detaching with EDTA-solution and re-suspended in IMDM medium (target cell amount 10 million/mL).

A small aliquot (50 µL) of the cell suspension was taken for LSC counting to estimate the total radioactivity to be injected

Step 2 (In-vivo Phase): 300 – 400 µL of cells suspension (containing about 2-3 million cells, and 300000 dpm in PBS) was injected into each mouse intraperitoneally (WT and MC1-R$^{e/e}$). Blood, feces, gallbladders and livers samples were collected for radioactivity (cholesterol) measurement after 24-48 hours post-injection.

*** Step 1 was conducted in collaboration with Maija Hollmen and Miro Viitala from Medicity Laboratory, Turku.

4.6 Radioactivity Measurements with Scintillation Counter

4.6.1 Plasma

Blood samples were collected into EDTA-coated tubes and then centrifuged at 4000 rpm for 10 min and the resulting plasma fractions were collected into separate tubes. An aliquot (100 µL) of the plasma was transferred into LSC tube and 4 mL of Optiphase Hisafe 3 LSC cocktail was added.

Samples were mix and allowed to stand for 60 min RT before measuring in a liquid scintillation counter (WALLAC 1410 LSC).

4.6.2 Liver

The whole liver was harvested, weighed and approximately 50 mg piece was transferred into a 20 mL scintillation vial. 1 mL SOLVABLE (Perkin Elmer) was added and the tube was heated in an incubator at 50 - 60 °C for 60 min. Sample was cooled to RT and 0.2 mL of 30% H_2O_2 in two aliquots of 0.1 mL was added and then the tubes were transferred to the incubator (50 °C) for 30 min to complete decolourisation. 12 mL of LSC cocktail was added to sample then allowed to temperature and light adapt for at least one hour before counting.

4.6.3 Feces

Feces were carefully collected from the cages and weighed (wet and dry). Thereafter, 0.1 mL water was added to 20 mg of dried feces and rehydrated for 30 min in a 20 mL vial. 1.0 mL of SOLVABLE was added and incubated at 50 °C for 2 hours followed by the addition of 1.0 mL of isopropanol and incubated for 2 hours at 50 °C. 30% H_2O_2 (0.2 mL) was added dropwise and allowed to stand for 15 to 30 minutes at RT. Samples were transferred into 50 °C incubator for 1 hour and left to cool to RT afterwards. 16 mL of LSC cocktail was added to the sample vial. The samples were allowed to temperature and light adapt for 1 hour before counting.

4.7 Statistics

Ct values from RT-PCR reactions were transferred unto Excel file for analysis. Student's t-test was used to compare two different groups (normal distributed data) and for the comparison of multiple groups, two-way ANOVA was applied on a GraphPad Prism (GraphPad Prism 6 software) program to assess statistical differences between genotype and dietary groups for each target genes and housekeeping genes. For H^3-cholesterol radioactivity data, student's t-test (two-tailed) and Mann Whitney U test was used to determine statistical significance. Data are expressed as means ± SEM and statistical significance for all comparisons is assigned at $p<0.05$

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6. ABBREVIATIONS LIST

α-MSH – alpha melanocyte stimulating hormones

ABC – ATP binding cassette transporter

ACE Inhibitors - Angiotensin-Converting-Enzyme Inhibitors

ACTH – adrenocorticotropic hormone

AGRP – agouti related protein

ApoA – Apolipoprotein A

ApoB – Apolipoprotein B

ApoE – Apolipoprotein E

ARG1 - Arginase

cAMP – cyclic adenosine monophosphate

CCL-2 - chemokine ligand 2

CCR5 - C-C chemokine receptor type 5

CD206 – Mannose Receptor

CD62P - P-selectin

CETP - cholesteryl ester transfer protein

CHD – Coronary Heart Disease

CVD – Cardiovascular Disease

CX3CR1 - CX3C chemokine receptor 1

HDL - High-Density Lipoprotein

HFD – High Fat Diet

ICAM1 – Intercellular Adhesion Molecule 1

IDL - Intermediate-Density Lipoprotein

IL-1 – Interleukin 1

IHD - Ischaemic Heart Disease

IP3 – inositol triphosphate

INFγ – Interferon gamma

KO – Knock Out

LDL - Low-Density Lipoprotein

LDL-r – Low Density Lipoprotein Receptor

LXRa – Liver X Receptor Alpha

LXR-RXR - Liver X Receptor–Retinoid X Receptor

MC1R – Melanocortin-1 Receptor

MMPs - matrix metalloproteinases

MYD88 - myeloid differentiation primary response protein 88

NOS – nitric oxide synthase

NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells

PECAM1 - Platelet Endothelial Cell Adhesion Molecule 1

PKC - phosphokinase C

PVD - Peripheral Vascular Disease

POMC – propopiomelanocortin

qPCR – Quantitative PCR

RT - Room Temperature

RCT - reverse cholesterol transportation

SR-BI - scavenger receptor class B type I

SREBP - Sterol Regulatory Element-Binding Proteins

TGFβ1 – Transforming growth factor beta

TNF-α - Tumour necrosis factor alpha

TLR - Toll-like receptor

VCAM-1 - vascular cell adhesion molecule-1

VLDL - Very Low Density Lipoprotein

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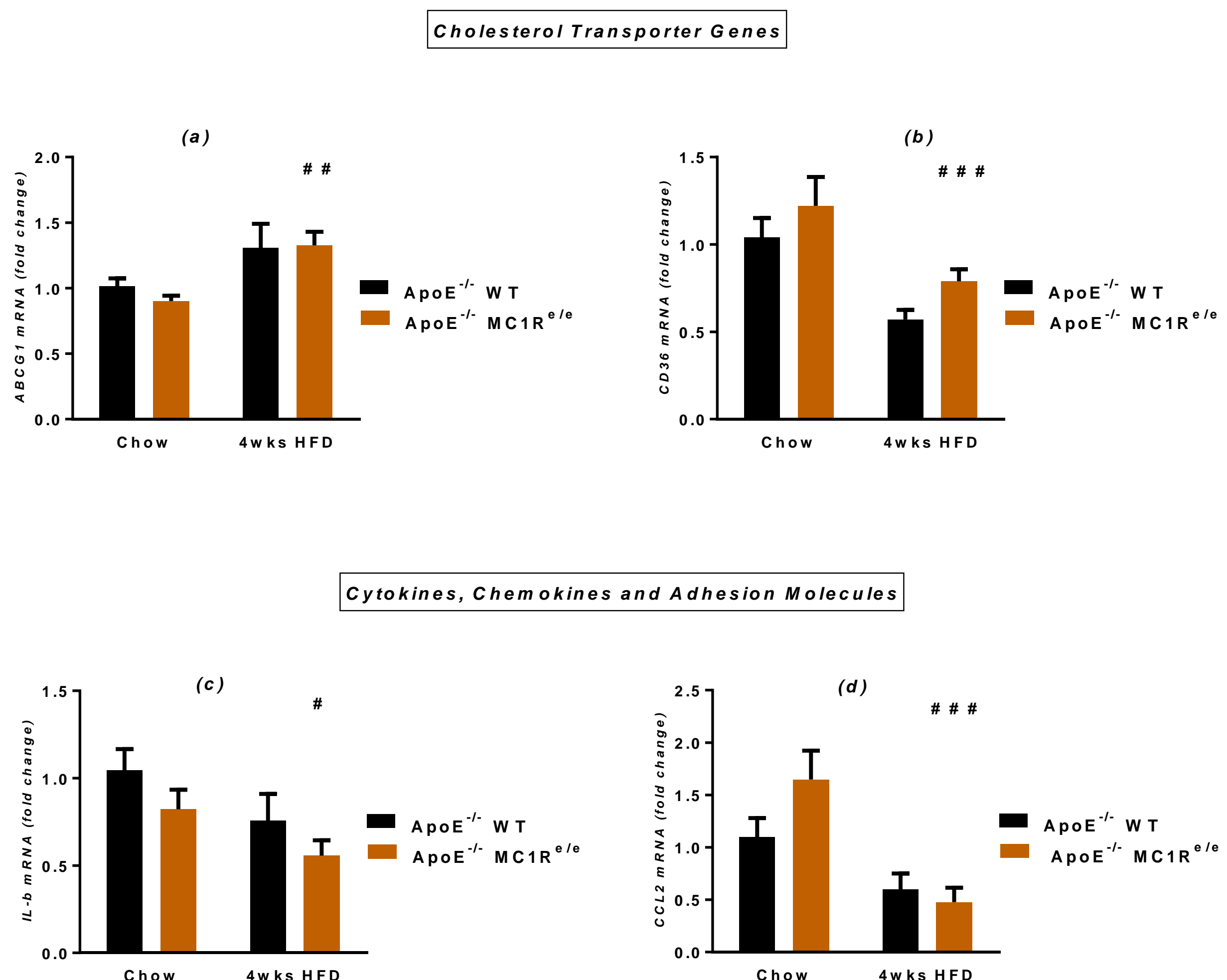
8. APPENDICES

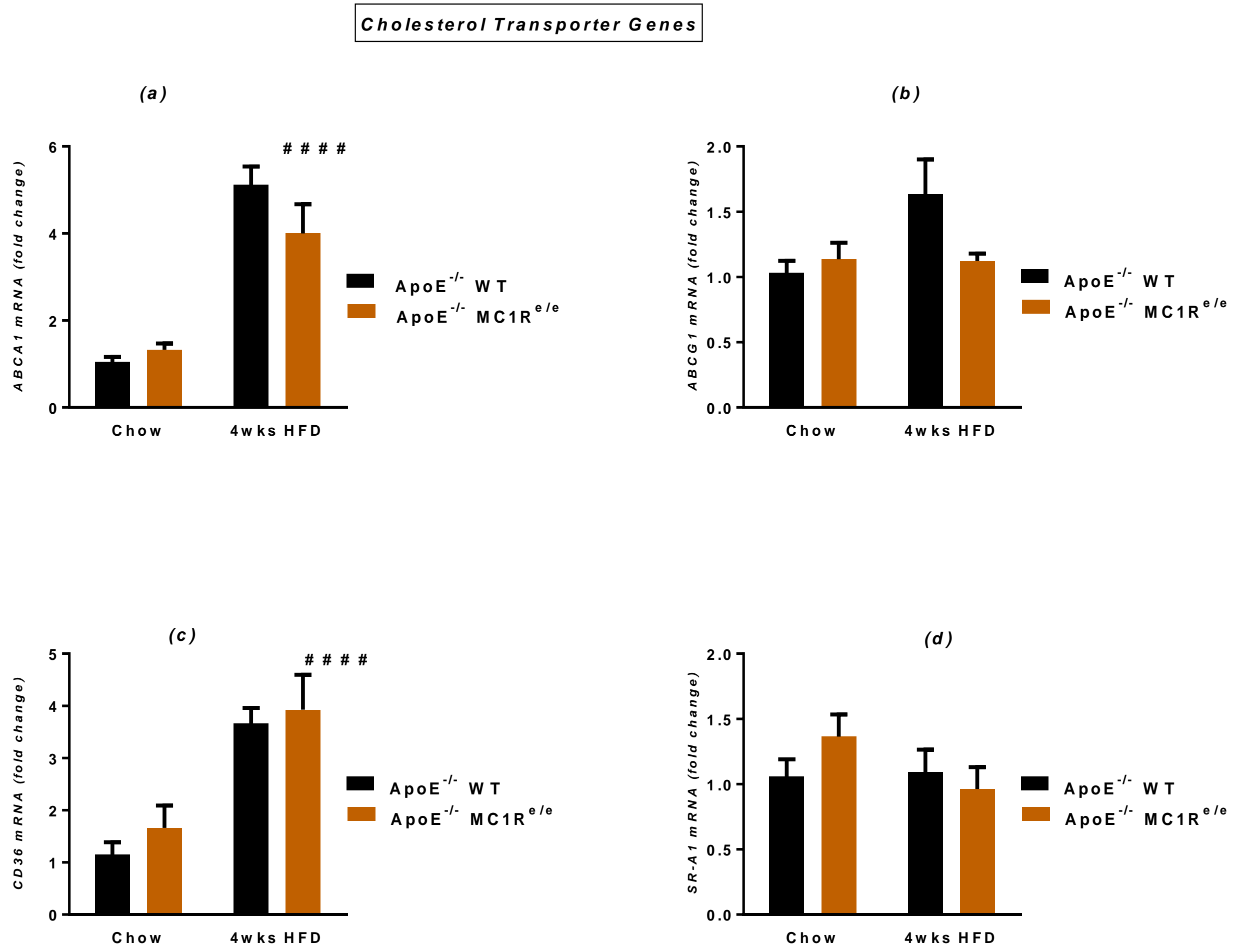


Supplemental Fig. 1: Cholesterol Transporters and Cytokines Gene in the Spleen of Mice

Cholesterol transporters and cytokines gene expression in the spleen of chow and 4WKs HFD mice.

(a) *ABCG1 (b) CD36 (c) IL-1β and, (d) CCL-2 mRNA expression in spleen samples of both genotypes and diet groups of mice.*

(# P < 0.05; ## P < 0.01; ### P < 0.001= dietary significance); n=8 per group.



Supplemental Fig. 2: Cholesterol Transporters and Scavenger Receptors in the Bone Marrow

Cholesterol transporters and scavenger receptor gene expression in the bone marrow of chow and 4wks HFD mice. (a) ABCA1 (b) ABCG1 (c) CD36 and, (d) SR-A1 mRNA expression in bone marrow samples of both genotypes and diet groups of mice.

(#### P < 0.0001= dietary significance); n=8 per group.