The effects of transgenic melanocortin overexpression in atherosclerotic mice

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Atherosclerosis is a chronic inflammatory disease of the arteries and a common cause of death worldwide. The disease is initiated by endothelial dysfunction that allows the transport of leukocytes and low-density lipoprotein (LDL) into the vessel wall where they form plaques. They impair the normal function of an artery. The melanocortin system is an endogenous modulatory system consisting of α-, β- and γ-melanocyte-stimulating hormones (α-, β- and γ-MSH), and corticotropin (ACTH); five melanocortin receptors, named MC1R-MC5R; and their antagonists, agouti and agouti-related peptide. Short-term melanocortin treatment alleviates vascular inflammation and dysfunction in atherosclerosis but, for now, no study has evaluated the long-term effects of the melanocortin system activation on atherosclerosis progression.

The aim of this study was to evaluate the effects of the transgenic melanocortin system activation in a mouse model of atherosclerosis. It was hypothesised that trangenic overexpression of the melanocortin peptides limits the progression of atherosclerosis. Low-density lipoprotein receptor-deficient (*Ldlr*$^{-/-}$) mice overexpressing α- and γ_3-MSH and their wildtype *Ldlr*$^{-/-}$ controls were fed either a regular CRM diet or high-fat and –sugar Western-style diet for 16 weeks. During this time, their body weight and food consumption were monitored weekly. After the diet intervention, the aortae were collected for *ex vivo* wire-myograph analysis of the functional and mechanical properties of the aorta. In addition, the plaques in the aortic root and arch were characterised by histological and immunohistochemical stainings, and the expressions of inflammatory mediators were studied by reverse-transcription polymerase chain reaction (RT-qPCR).

We found that on Western diet the melanocortin overexpression limited the plaque formation in the aortic arch and the expression of inflammatory cytokines (*Ccl2*, *Ccl5* and *Il6*) that contribute to the pathogenesis of atherosclerosis. In addition, the melanocortin overexpression alleviated the α_1-adrenoceptor-mediated vasoconstriction and enhanced the endothelium-dependent dilation. Thus, the transgenic melanocortin overexpression improved the function of the aorta. These results show for the first time that the transgenic activation of the melanocortin system limits the progression of atherosclerosis by alleviating inflammation, plaque formation and arterial dysfunction.

Keywords: atherosclerosis, melanocortin, inflammation, cardiovascular

ABBREVIATIONS

ACh	Acetylcholine
ACTH	Corticotropin
AgRP	Agouti-related peptide
ANOVA	Analysis of variance
ARC	Arcuate nucleus of the hypothalamus
CCL	Chemokine ligand
CD	Cluster of differentiation
CNS	Central nervous system
CRH	Corticotropin-releasing hormone
CRM	Certified reference material
CVD	Cardiovascular disease
HPA	Hypothalamus-pituitary-adrenal
IL	Interleukin
LDL	Low-density lipoprotein
L-NNA	N^{ω}-Nitro-L-arginine
MCR	Melanocortin receptor
MHC	Major histocompatibility complex
MSH	Melanocyte-stimulating hormone
NF-κB	Nuclear factor κB
NO	Nitric oxide
NOS	Nitric oxide synthase
OE	Overexpression
PC	Prohormone convertase
POMC	Pro-opiomelanocortin
RT-qPCR	Quantitative reverse-transcription polymerase chain reaction
SEM	Standard error of the mean
SNP	Sodium nitroprusside
UV	Ultraviolet
WAT	White adipose tissue
WT	Wildtype

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1 INTRODUCTION

Acute complications in cardiovascular diseases (CVDs) cause more than 30% of all deaths, making them the number one killer worldwide (World Health Organization 2015, Mozaffarian, Benjamin et al. 2015). In Finland in 2010, the age-standardised death rate of cardiovascular diseases was more than 300 per 100 000 citizens, which means that every fifth citizen died of coronary artery disease (Statistics Finland 2011). The most acute complications of CVDs originate from atherosclerosis, the formation of lipid-rich plaques in the vessel wall (World Health Organization 2015). Atherosclerosis is an inflammatory disease of the middle- and large-sized arteries and on the basis of current understanding it is initiated by endothelial dysfunction (Moore, Tabas 2011). Several factors have been suggested to cause endothelial damage, among others, elevated cholesterol and triglyceride concentrations in the blood (American Heart Association 2014). Moreover, a major risk factor for atherosclerosis is metabolic syndrome, which includes the sum of obesity, high blood pressure, low HDL cholesterol, high triglyseride levels and impared glucose homeostasis.

A growing body of evidence recognises the beneficial effects of a peptide system called the melanocortin system (Catania, Gatti et al. 2004, Patel, Leoni et al. 2010). This system consists of the melanocortin peptides, α-, β- and γ-melanocyte-stimulating hormones (α-, β- and γ-MSH), and corticotropin (ACTH); five melanocortin receptors, named MC1R-MC5R (Mountjoy, Robbins et al. 1992); and their antagonists, agouti and agouti-related peptide (AgRP) (Gantz, Fong 2003, Cortes, Navarro et al. 2014).

A wealth of evidence has recognised the benefits of the melanocortin system activation on cardiovascular and inflammation regulation both *in vitro* and *in vivo* (Brzoska, Luger et al. 2008, Catania, Lonati et al. 2010, Leoni, Patel et al. 2008, Leoni, Voisin et al. 2010, Patel, Montero-Melendez et al. 2011, Rinne, Nordlund et al. 2013, Rinne, Silvola et al. 2014, Schaible, Steinstrasser et al. 2013). Recent studies have showed that α-MSH and its analogue, melanotan II (MT-II), evoke anti-inflammatory and vasoactive effects both in endothelial cells and in a mouse model of atherosclerosis (Rinne, Silvola et al. 2014, Yang, Zhang et al. 2015). On the other hand, deficient MC1R function disturbs the vascular endothelial function and increases arterial stiffness both in mice and humans (Rinne, Ahola-Olli et al. 2015). The vasoprotective effects arise from the

augmentation of nitric oxide (NO) availability (Davignon, Ganz 2004, Rinne, Nordlund et al. 2013). The alleviation of inflammation stems, on one hand, from the inhibition of nuclear factor κB related gene products (Manna, Aggarwal 1998, Yang, Zhang et al. 2015), such as pro-inflammatory cytokines, their receptors and adhesion molecules (May, Ghosh 1998) and, on the other hand, from the activation of anti-inflammatory processes (Holloway, Durrenberger et al. 2015). Apart from MC1R, MC3R is also instrumental in mediating the anti-inflammatory and vasoprotective effects of melanocortins. Pharmacological treatment with an MC3R analogue attenuates cell adhesion, emigration and chemokine generation, while deficiency in *Mc3r* manifests in increased extravasation and upregulation of inflammatory markers (Leoni, Patel et al. 2008).

Although the beneficial effects of the melanocortin system on immune and cardiovascular regulation have been clearly characterised (Catania, Gatti et al. 2004, Catania 2007, Catania, Lonati et al. 2010, Samuelsson 2014, Ahmed, Kaneva et al. 2014), the therapeutic potential of long-term melanocortinergic activation remains to be determined. To study the long-term melanocortin system activation in atherosclerosis, an atherosclerotic low-density lipoprotein receptor-deficient (*Ldlr*$^{-/-}$) mouse model that overexpresses α- and γ_3-MSH was created and the progression of atherosclerosis in this transgenic disease model was evaluated. In this study, the trangenic α- and γ_3-MSH overexpression (OE) limited the progression of vascular dysfunction, inflammation and plaque formation. This study shows for the first time that transgenic α- and γ_3-MSH-OE alleviates the progression of murine atherosclerosis.

2 REVIEW OF THE LITERATURE

2.1 Melanocortin system

2.1.1 Pro-opiomelanocortin

Melanocortins, α-, β- and γ-MSH, and ACTH, are biologically active, endogenous peptides that are cleaved from the common precursor molecule pro-opiomelanocortin (POMC) (Smith, Funder 1988). γ-MSH comes in three species of which γ_1-MSH and γ_2-

MSH have nearly identical amino acid sequences, while γ_3-MSH is a larger peptide than γ_1- and γ_2-MSH. POMC is cleaved to active melanocortins by calcium-dependent subtilisin-like endoproteases, called prohormone convertases PC1/3 and PC2 (Fig. 1 and 2). The *POMC* gene is conserved among all mammalian species and it consists of two introns and three exons (Autelitano, Lundblad et al. 1989). *POMC* is mainly expressed in the pituitary, but also in the arcuate nucleus of the hypothalamus (ARC), in the medulla and in many peripheral tissues, such as skin, kidney and liver (Wardlaw 2011, Autelitano, Lundblad et al. 1989). *POMC* is regulated by several factors, including corticotropin-releasing hormone (CRH), serotonin, adrenalin, oxytocin, angiotensin II and bradykinin, which stimulate *POMC* transcription, and γ-aminobutyric acid and glucocorticoids, which inhibit *POMC* (Smith, Funder 1988, Autelitano, Lundblad et al. 1989).

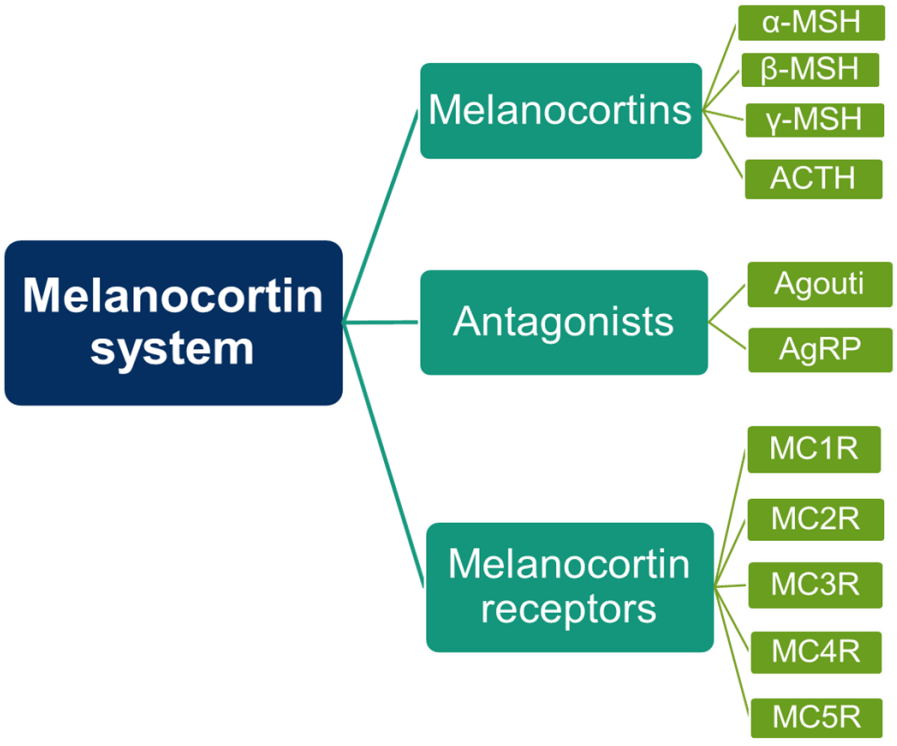

Fig. 1. The melanocortin system consists of endogenous melanocortins and antagonists, and melanocortin receptors that mediate the responses into the cell.

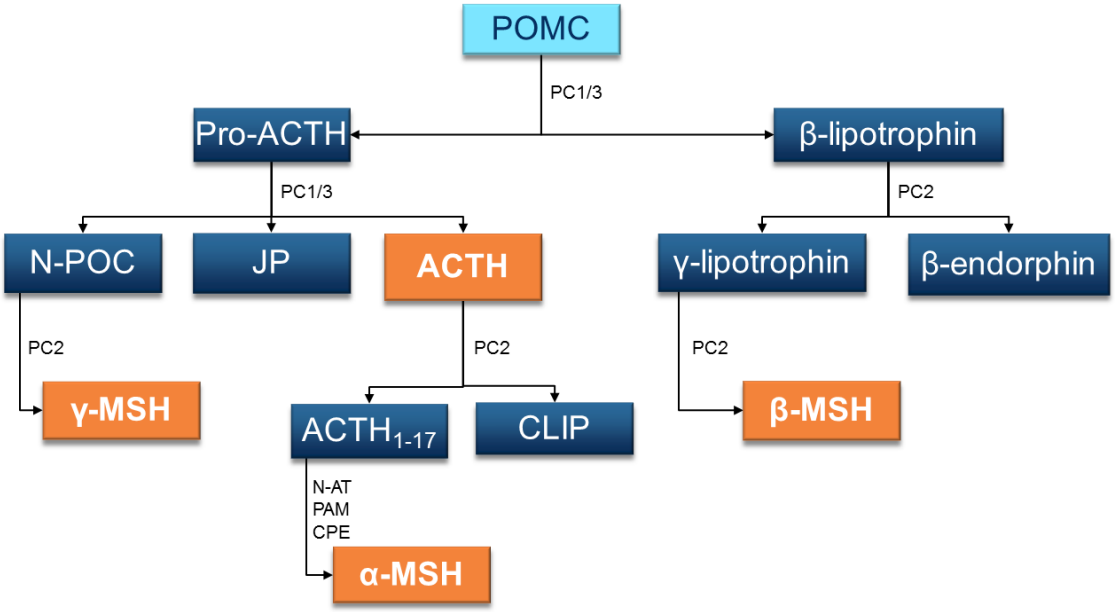

Fig. 2. POMC is posttranscriptionally processed to active melanocortins. Melanocortins peptides, α-, β- and γ-MSH, and ACTH, are enzymatically cleaved from a common prohormone precursor protein POMC. *N*-POC, *N*-terminal peptide; JP, joining peptide; *N*-AT, *N*-acetyltransferase; PAM, peptidylglycine α-amidating mono-oxygenase; CPE, carboxypeptidase; CLIP, corticotropin-like intermediate lobe peptide.

2.1.2 Melanocortin receptors

Melanocortins mediate their effects via five melanocortin receptors (MC1R-MC5R). MCRs are the smallest stimulatory G-protein coupled receptors (GPCR) identified to date with a short extracellular *N*-terminus and intracellular *C*-terminus. The melanocortin system is an exceptional peptide system because it contains both endogeneous agonists as well as antagonists although this chapter mainly focuses on MCRs and their agonists.

MC1R is a classic melanocortin receptor that has a high affinity for α-MSH and ACTH (Mountjoy, Robbins et al. 1992, Chhajlani, Wikberg 1992). MC1R is expressed in dermal fibroblasts, keratinocytes and melanocytes, but also in the immune and endothelial cells. In the skin, the ligand-receptor interaction stimulates the synthesis of eumelanin and induces pigmentation when the skin is exposed to ultraviolet (UV) light (Abdel-Malek, Swope et al. 1995, Hunt, Todd et al. 1994, Robbins, Nadeau et al. 1993). This biological response is beneficial because it protects the skin from UV light-induced genomic damage (Rees 2003). UV light provokes the production of α-MSH in the skin

where α-MSH acts in a paracrine manner to alleviate inflammation. Pigmentation is altered by MC1R mutations and polymorphisms (Robbins, Nadeau et al. 1993, Koppula, Robbins et al. 1997). Along with skin, MC1R is present in the cells of the immune system (Star, Rajora et al. 1995, Bhardwaj, Becher et al. 1997, Neumann Andersen, Nagaeva et al. 2001, Catania, Rajora et al. 1996, Becher, Mahnke et al. 1999), playing an important role in immunomodulation. In addition, weak MC1R signalling has been linked to defects in arterial vasodilatation and distensibility (Rinne, Ahola-Olli et al. 2015).

MC2R binds only ACTH, which is why it is also called the ACTH receptor. MC2R is expressed in the adrenal cortex, where it stimulates the synthesis of corticosteroids, such as cortisol in humans and corticosterone in rodents (Tsigos, Arai et al. 1993) that regulate glucose metabolism and immune responses (Xia, Wikberg 1996, Lefkowitz, Roth et al. 1970, Mountjoy, Robbins et al. 1992). MC2R is also expressed in adipocytes in mice, where it mediates lipolysis (Boston, Cone 1996).

MC3R (Gantz, Konda et al. 1993) is mainly expressed in the hypothalamus in the central nervous system (CNS) (Roselli-Rehfuss, Mountjoy et al. 1993), but also in a variety of other tissues, such as, in the heart, gut, placenta (Gantz, Konda et al. 1993), macrophages (Lam, Perretti et al. 2006) and B cells (Cooper, Robinson et al. 2005). MCR3 is the only MCR subtype that responds highly to γ-MSH (Roselli-Rehfuss, Mountjoy et al. 1993). However, MC3R is also activated by other melanocortin peptides. MC3R contributes to immunomodulation (Getting, Gibbs et al. 1999, Getting, Allcock et al. 2001), energy homeostasis and feeding behaviour (Marks, Hruby et al. 2006).

Moreover, MC4R is present in the CNS (Mountjoy, Mortrud et al. 1994, Gantz, Miwa et al. 1993). However, compared to MC3R, MC4R is more wide-spread within the CNS (Mountjoy, Mortrud et al. 1994). MC4R contributes to the central regulation of cardiovascular dynamics (Dunbar, Lu 2000, Matsumura, Tsuchihashi et al. 2002), energy homeostasis and feeding behaviour (Huszar, Lynch et al. 1997). MC4R is activated by α- and ß-MSH and ACTH, but also by γ-MSH although with lower affinity.

MC5R is expressed in a variety of peripheral tissues, such as thymus, spleen and exocrine glands (Gantz, Shimoto et al. 1994, Labbe, Desarnaud et al. 1994). This receptor subtype contributes to the lipid mobilisation and synthesis and secretion of exocrine products (Boston, Cone 1996, Chhajlani, Muceniece et al. 1993, Chen, Kelly et al. 1997). MC5R may also have a role in inflammation control because it is expressed in B cells (Buggy 1998), T cells (Taylor, Namba 2001) and mast cells (Artuc, Grutzkau et al. 1999). MC5R responds to α-MSH that has higher affinity than β-MSH and ACTH. γ-MSH activates MC5R, too, but has weaker affinity than the above-mentioned agonists.

The distribution, function and endogeneous agonists and antagonists of five MCR subtypes are presented in Table 1.

Table 1. The properties of the melanocortin receptor subtypes (modified from (Caruso, Lagerstrom et al. 2014)). GI-tract, gastrointestinal tract.

Receptor subtype	Distribution	Function	Ligand affinity	Endogenous antagonists
MC1R	Skin, immune cells, glial cells and astrocytes	Pigmentation, anti-inflammation, nociseption	α-MSH > β-MSH ACTH > γ-MSH	Agouti
MC2R	Adrenal cortex, skin, adipose tissue, bone cells	Steroidogenesis, lipolysis	ACTH	Agouti
MC3R	CNS, heart, GI-tract, kidney, immune cells	Energy homeostasis, food intake, anti-inflammation, natriuresis	α-MSH = β-MSH = γ-MSH = ACTH	AgRP
MC4R	CNS	Energy homeostasis, food intake, anti-inflammation, neuroprotection	α-MSH = β-MSH = ACTH >> γ-MSH	Agouti, AgRP
MC5R	Skin, spleen, lung, GI-tract, bone marrow, adipose tissue	Exocrine secretion, lipid mobilisation	α-MSH > β-MSH = ACTH > γ-MSH	Agouti

2.1.3 Synthetic melanocortin peptides

The synthetisation of melanocortin-like peptides has enabled the recognition of the amino acids that mediate the receptor-ligand interaction in MSHs (Hruby, Sawyer et al.

1980, Sawyer, Hruby et al. 1982). This core heptapeptide, His-Phe-Arg-Trp, is crucial for mediating the cellular responses of MSHs because MCRs recognise and bind to this structure (Haskell-Luevano, Sawyer et al. 1996, Sawyer, Hruby et al. 1982) (Fig. 3).

Fig. 3. The amino acid sequences of endogenous α-, β- and γ-melanocyte stimulating hormones (α-, β- and γ-MSH, respectively). All MSHs share a core amino acid sequence that is represented in blue. This core sequence is crucial for the ligand-receptor interaction (modified from (Humphreys, Lin 1988)).

Afamelanotide, also known as melanotan I or [Nle^4-D-Phe^7]-α-MSH, is one of the first synthesized α-MSH analogues (Sawyer, Sanfilippo et al. 1980). Compared with endogenous α-MSH, afamelanotide has a prolonged bioactivity because it is more resistant to enzymatic degradation (Sawyer, Sanfilippo et al. 1980, Hadley, Anderson et al. 1981). Similar to α-MSH, afamelanotide protects the skin from UV radiation. Due to its photoprotective action, afamelanotide has recently been approved for the treatment of erythropoietic protoporphyria (Minder 2010).

Another α-MSH analogue, MT-II, is as potent as its parent compound afamelanotide in inducing skin pigmentation. Unlike afamelanotide, MT-II is able to cross the blood-brain barrier (BBB) and move to the CNS, where it acts as an anorexigenic agent, i.e. it reduces food intake. Due to the tanning and anorexigenic effects, MT-II is also called as a “Barbie drug” and used unlicensed for cosmetic reasons. As a side effect, MT-II causes unintentional erections in men. This observation led to the development of PT-141 or bremelanotide that has been approved for the treatment of female sexual dysfunction (Diamond, Earle et al. 2006). Because of their central role in the regulation of energy homeostasis and food intake, several melanocortin agonists, both non-

selective MC3R/MC4R and selective MC4R agonists, are currently being investigated for the treatment of obesity (Fani, Bak et al. 2014).

The amino acid sequences of α-MSH and its synthetic analogues MT-I and MT-II are presented in Fig. 4.

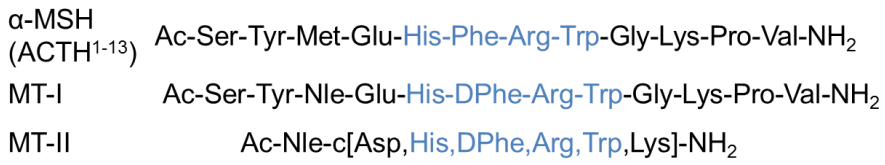

Fig. 4. The amino acid sequences of endogenous α-MSH and its synthetic analogues MT-I and MTII. The core sequence of MSHs is presented in blue (Hadley, Dorr 2006).

2.2 Physiological functions of the melanocortin system

Over the past years, it has been demonstrated that melanocortins play an important role in maintaining health and homeostasis (Cone 2006) (Fig. 5). The cells are constantly under intrinsic or external stressors, such as pathogens or UV radiation, which disturb the cellular homeostasis. The stress response is mainly mediated via the hypothalamus-pituitary-adrenal (HPA) axis. When it is activated, CRH is produced in the hypothalamus, from where it is transported to the portal circulation (Popa, Fielding 1933, Harris 1948) and to the anterior pituitary. There CRH promotes the synthesis of POMC (Affolter, Reisine 1985). POMC products, ACTH and MSHs, are secreted to the systemic circulation, where they are delivered to their target tissues. In the periphery, melanocortins bind to MCRs and induce the production of mediators, for example, glucocorticoids from the adrenal gland. Then, the mediators alleviate the stress response and also convey negative feedback to the pituitary and hypothalamus. (Slominski, Wortsman et al. 2000)

At a cell level, binding of a melanocortin ligand to its receptor activates adenylyl cyclase (AC) (Sawyer, Hruby et al. 1982, Tatro, Entwistle et al. 1990), which converts ATP to cyclic adenosine monophosphate (cAMP). cAMP is a secondary messenger that activates protein kinase A (PKA) (Mountjoy, Robbins et al. 1992). PKA regulates the phosphorylation of cAMP response element-binding protein (CREB) that interacts with

cAMP response element (CRE) at the promoter area and activates transcription of an anti-inflammatory gene, for example, *CRH* (Sarkar, Legradi et al. 2002).

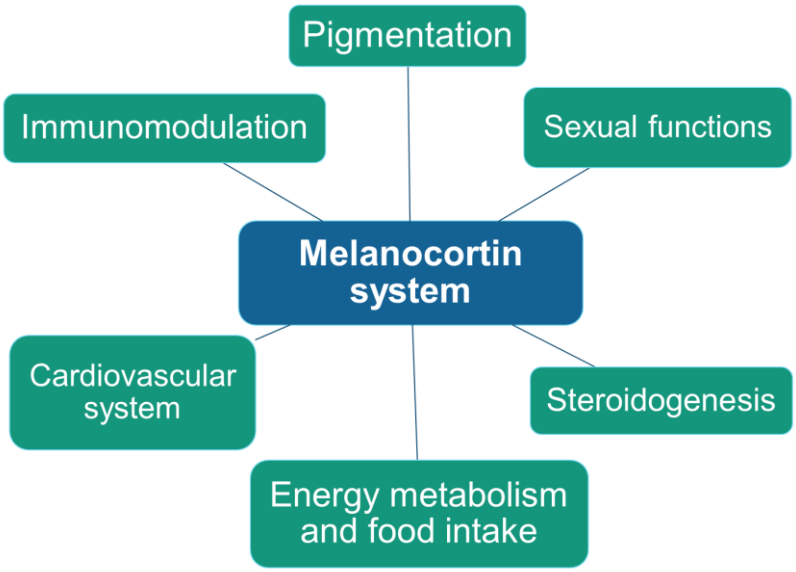

Fig. 5. Melanocortin system regulates several physiological functions in order to maintain homeostasis.

Some of the key functions of the melanocortin system potentially related to atherosclerosis are discussed in this chapter.

2.2.1 Energy homeostasis

Today, obesity is a worldwide health issue and a risk factor for cardiovascular and other diseases (Nguyen, Lau 2012). Obesity increases the risk for diabetes and the hyperglycemia related to diabetes increases the risk for atherosclerosis. The central melanocortin system is an important regulator of the energy homeostasis (Nuzzaci, Laderriere et al. 2015). The regulatory centre of this system lies in the ARC. This nucleus contains two types of neuron populations: the POMC and the AgRP/neuropeptide Y (NPY) neurons (Cowley, Pronchuk et al. 1999) that mediate opposing effects. AgRP/NPY neurons express orexigenic molecules such as NPY and AgRP, thus increasing appetite (Clark, Kalra et al. 1984, Stanley, Leibowitz 1984, Fani, Bak et al. 2014), whereas POMC neurons express anorexigenic signals, such as POMC and cocaine- and amphetamine-regulated transcript, hence mediating the satiety

response (Cowley, Pronchuk et al. 1999, Fani, Bak et al. 2014). Together, these neurons of the central melanocortin system regulate the food intake and metabolic status.

A number of studies demonstrate the relevance of the melanocortin precursor POMC in the regulation of energy metabolism (Nuzzaci, Laderriere et al. 2015). Deficiencies in *POMC* or the deletion of *Pomc* results in obesity in humans and in mice, respectively (Yaswen, Diehl et al. 1999, Greenman, Kuperman et al. 2013). On the other hand, OE of POMC peptides protects from obesity (Savontaus, Breen et al. 2004). POMC neuron activity and *POMC* expression are modulated by several peripheral metabolic signals (Jeong, Kim et al. 2014). These signals include blood glucose, pancreas-derived insulin and adipose tissue-originated leptin. In response to these signals, *POMC* is upregulated and cleaved to melanocortin peptides. As a result, POMC product α-MSH decreases appetite via binding to MC4R in the paraventricular hypothalamus (Schwartz, Seeley et al. 1997, Fani, Bak et al. 2014). AgRP/NPY neurons, on the other hand, are negatively regulated by insulin or leptin.

An essential mediator of the central melanocortin system is MC4R. The administration of an MC4R antagonist or the deletion of *Mc4r* restrains the connection between the POMC neurons and the anorexic neurons, which causes obesity (Fan, Boston et al. 1997, Huszar, Lynch et al. 1997). On the other hand, the activation of MC4R in anorexic neurons reduces appetite (Fan, Boston et al. 1997). These mouse data are consistent with the human data, because in humans, rare loss-of-function mutations in *MC4R* are often associated with obesity (Farooqi 2008, Rene, Le Gouill et al. 2010). MC3R plays also a role in the control of energy balance, but the correlation between *MC3R* variants and obesity is less evident than that of *MC4R* (Li, Joo et al. 2000).

In addition to several satiety-related molecules, energy balance is modulated by inflammatory mediators. In rats, endotoxin LPS promotes *POMC* expression in the CNS and reduces food intake. The authors speculate that the cytokine-release induced by LPS would increase plasma leptin level, which in turn, activates POMC neurons, and further, α-MSH production. (Sergeyev, Broberger et al. 2001) Moreover, high-fat diet increases the expression of pro-inflammatory cytokines and nuclear factor κB (NF-κB) pathway genes in the hypothalamus. This response is independent of the weight gain because 1 to 3 days on the diet already activates the hypothalamic inflammation. Furthermore, one

week on the high-fat diet reduces the number of POMC neurons in mice and in rats. The rodent data are consistent with human magnetic resonance imaging findings, which demonstrate that obesity is associated with mediobasal hypothalamic injury. (Thaler, Yi et al. 2012)

2.2.2 Cardiovascular system

The melanocortin system regulates the cardiovascular system, including blood pressure and endothelial function. The effects of melanocortins on blood pressure regulation are complex because these peptides may have either hypotensive or hypertensive effects depending on the dose and the site of action (Callahan, Kirby et al. 1984, Lymangrover, Buckalew et al. 1985).

The primary melanocortin regulating blood pressure is γ-MSH. Deficiency in γ-MSH, caused by genetic deletion of PCs that process POMC to γ-MSH, leads to salt-sensitive hypertension (Mayan, Ling et al. 1996). On the other hand, an acute administration of γ-MSH elevates the blood pressure and heart rate temporarily (Callahan, Kirby et al. 1988, Ni, Butler et al. 2006). However, the hypertensive effects of γ-MSH are not mediated via MC3R but via FMRFamide gated sodium channels (Versteeg, Van Bergen et al. 1998, Ni, Butler et al. 2006). Furthermore, α- and γ_3-MSH-OE elevates the blood pressure, but not the heart rate (Rinne, Harjunpää et al. 2008). These effects are likely mediated via central sympathetic stimulation (Callahan, Kirby et al. 1988, Ni, Butler et al. 2006).

While γ-MSH mediates its effect via FMRFamide gated sodium channels, α-MSH exerts its centrally mediated cardiovascular effects via MC4R activation (Ni, Butler et al. 2006). Intracerebroventricularly administered α-MSH increases the blood pressure and heart rate via sympathetic activation (Ni, Butler et al. 2006), whereas α-MSH administration into nucleus tractus solitarii in medulla decreases blood pressure and heart rate (Tai, Weng et al. 2007). α-MSH analogue [Nle^4-D-Phe^7]-α-MSH shows distinct effects in mice and in rats when administered intravenously. In rats, there are no cardiovascular effects, while mouse studies show an elevation in both heart rate and blood pressure (Van Bergen, Kleijne et al. 1997, Rinne, Tikka et al. 2012).

In addition to its central actions, α-MSH regulates the cardiovascular system in the periphery. In the vascular endothelium, α-MSH enhances NO synthesis, thus inducing vasorelaxation and protecting the endothelium (Rinne, Nordlund et al. 2013). These beneficial effects are disrupted in *Mc1r*$^{e/e}$ mice that lack functional Mc1r (Rinne, Ahola-Olli et al. 2015). Mc1r deficiency hardens the aorta, impairs the endothelium-dependent vasodilatation and predisposes to vascular inflammation (Rinne, Ahola-Olli et al. 2015). Thus, the beneficial effects of α-MSH on vascular endothelium seem to be mediated via MC1R.

2.2.3 Immune system

Already in 1950, Hench observed that ACTH alleviates the symptoms of rheumatoid arthritis (Hench 1950). Since, the therapeutic potential of the melanocortin treatment has expanded to include not only gouty arthritis but also other inflammatory disorders. Today, the melanocortin treatment has been documented to be beneficial in several preclinical models of inflammatory diseases (Catania, Lonati et al. 2010). Moreover, in clinic, ACTH is already being used for the treatment of multiple sclerosis relapses.

The melanocortin system has anti-inflammatory, immunomodulatory and anti-microbial properties in the immune cells (Catania 2008) and almost all types of leukocytes express MCRs (Catania 2007) (Fig. 6). This chapter discusses the mechanisms by which melanocortins mediate their immunomodulatory actions and the role of melanocortin system in specific cells of the immune system that relate to atherosclerosis.

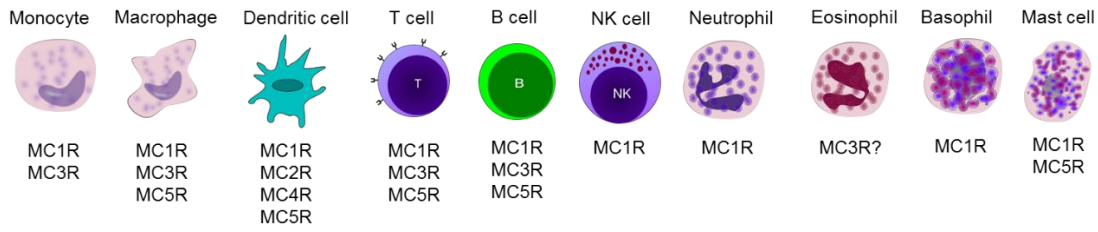

Fig. 6. The melanocortin receptor expression in the immune cells. Melanocortin receptors are widely expressed in the immune system. Clipart pictures of the immune cells retrieved from www.clker.com.

2.2.3.1 Anti-inflammatory signalling pathways of the melanocortin system

Inflammatory response is a defence mechanism that restricts the tissue damage and initiates the healing process. Inflammation responses can be classified as acute and chronic inflammatory responses. In acute inflammation, neutrophils dominate the inflammatory site and secrete proteins from their granules into the surrounding tissue. The later stage, when the neutrophils have been replaced by monocytes, lymphocytes and plasma cells, is called the chronic inflammatory response and is mediated by adaptive immunity. Eventually, the inflammatory response leads to resolution and healing. Sometimes the inflammatory response can be too extensive or chronic. Therefore, anti-inflammatory mechanisms are needed in order to resolve the inflammation. The melanocortin system alleviates inflammation particularly at its early stage by inhibiting the production of cytokines and growth factors, cytokine receptors, leukocyte adhesion molecules and stress proteins.

Melanocortins mediate their anti-inflammatory effects via three main pathways.

1. Melanocortins can modulate the action of peripheral immune cells via descending anti-inflammatory pathways and via HPA axis.
2. Melanocortin can inhibit the immune response by decreasing the secretion of inflammatory mediators by the immune cells.
3. Melanocortins can inhibit the inflammation locally in the CNS.

Next, these pathways are discussed in more detail.

In addition to the HPA axis that was discussed earlier, melanocortins mediate their anti-inflammatory effects via descending pathways (Macaluso, McCoy et al. 1994). When melanocortins bind to the central MC3R or MC4R, the ligand-receptor interaction activates the cholinergic and brain-spinal cord adrenergic circuits. These pathways mediate the anti-inflammatory effects to the peripheral immune cells via nicotinic and β_2 adrenergic receptors, respectively (Matsumura, Tsuchihashi et al. 2002). Animal studies using spinal cord transections highlight the relevance of an intact spinal cord. In transectioned animals, the melanocortin-induced anti-inflammatory effects remain lower compared to intact animals. The same effect is observed when peripheral β_2 receptor antagonist, for example, propranolol is administrated (Macaluso, McCoy et al. 1994). These evidence show that the immunomodulation evoked by the melanocortin system is a sum of peripheral and central actions; thus, immunodulation is often more extensive in a living organism than *in vitro*.

Melanocortins can also act locally in the CNS by binding to the melanocortin receptors in the glia. Within the CNS, melanocortins alleviate the central inflammation and protect cells against neuronal damage. Such protection could be beneficial, for example, in the case of traumatic brain injury (Schaible, Steinstrasser et al. 2013).

At the transcriptional level, melanocortins mediate their effects via inhibition of the transcription factor NF-κB that is responsible for the activation of several inflammatory genes, including proinflammatory cytokines and chemokines, growth factors and inducible nitric oxide synthase (iNOS) (Fig. 7) (Manna, Aggarwal 1998). Normally, NF-κB is located in the cytoplasm, where it is bound to the inhibitory proteins of IκB family. However, when a cell is exposed to an inflammatory stimulus, such as, pathogen, chemokine or endotoxin from bacteria, IκB is phosphorylated and degraded. Then, free NF-κB is able to translocate to the nucleus, where it binds to the promoter area of an inflammatory gene activating its transcription. This cascade mediates an inflammatory response. But when melanocortins bind to MCRs, they inhibit the translocation of NF-κB to the nucleus and therefore, prevent the initiation of an inflammatory response. Moreover, glucocorticoids, induced by ACTH, activate the transcription of IκB that inhibits the translocation of NF-κB (Scheinman, Cogswell et al. 1995). Hence, melanocortins alleviate inflammation at an early state of an immune response. For this reason, the inhibition of NF-κB by melanocortin treatment appears as an ideal drug development target for a variety of inflammatory disorders (Catania, Lonati et al. 2010).

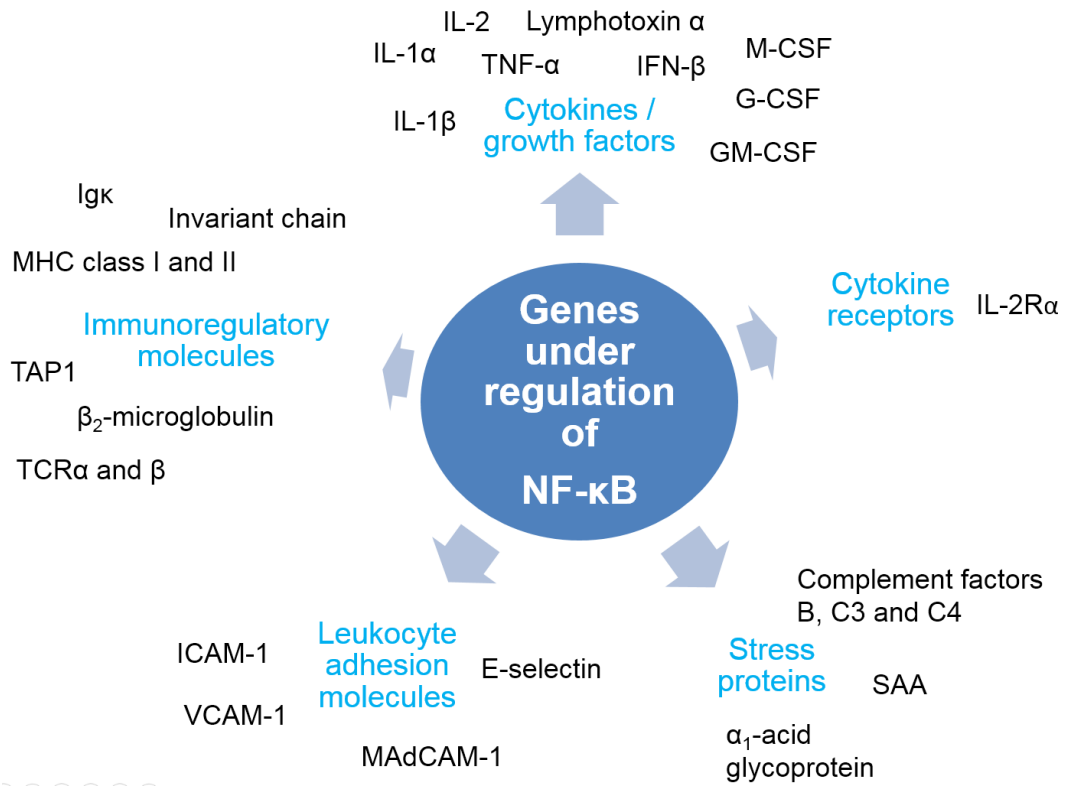

Fig. 7. Genes under regulation of NF-κB. NF-κB upregulates several inflammatory genes when a cell is exposed to an inflammatory stimulus, for example, bacterial lipopolysaccharide (LPS). Melanocortins alleviate inflammation by inhibiting NF-κB, and hence, NF-κB-related gene products.

2.2.3.2 Neutrophils

Neutrophils compose 40-75% of blood leukocytes in humans, making them the most common circulating leukocyte subtype. The migration of neutrophils is the first and most important step in the acute immune response. During the acute inflammation, neutrophils phagocytose and destroy the pathogens and wounded cells.

During the acute inflammation, neutrophils attach to the endothelial adhesion molecules and extravasate from the blood to the tissue. The extravasation is mediated by IL8 that binds to CXCR receptors on neutrophils. This interaction unbinds NF-κB from its inhibitory proteins and triggers the expression of inflammatory mediators as described earlier. Neutrophils express MC1R on their cell surface. MC1R/α-MSH interaction downregulates CXCR; and thus, α-MSH restrains the activation of acute neutrophil-driven immune response. (Manna, Sarkar et al. 2006)

2.2.3.3 Dendritic cells

Dendritic cells are the bridge between the innate and adaptive immunity. They recognise, phagocytose and destroy the pathogens. The destroyed pathogen fragments, also called as epitopes, are then presented to naïve CD (cluster of differentiation) 4^+ T cells in the lymph nodes. The antigen presentation is mediated via major histocompatibility complex (MHC) II molecule and T cell receptor (TCR) interaction. The antigen presentation enables the differentiation of $CD4^+$ T cells into Th_1, Th_2, Th_{17} or T_{reg} cells, depending on the surrounding cytokines.

In addition to MHC II and TCR, costimulatory molecules are needed for the antigen presentation and T cell survival. These molecules include CD86 and CD40 that are expressed on the surface of antigen presenting cells, including dendritic cells. Dendritic cells express MCR 1, 2, 4 and 5 subtypes. Binding of α-MSH to MC1R induces tolerogenic dendritic cells and expansion of regulatory T cells (Auriemma, Brzoska et al. 2012). α-MSH-mediated tolerance is characterised by decreased expression of costimulatory molecules (Auriemma, Brzoska et al. 2012). In addition, α-MSH downregulates the proliferation of Th_{17} effector cells (Auriemma, Brzoska et al. 2012). Together, α-MSH inhibits the progression of cell-mediated inflammation.

2.2.3.4 Monocytes and macrophages

Monocytes are circulating leukocytes. As they move to the inflamed tissue, they differentiate into macrophages. Their role is to destroy cells that are infected by microbes and present the epitopes to other immune cells. Activated macrophages also recruit other immune cells to the site of inflammation by secreting chemokines, i.e. chemotactic cytokines.

Melanocortins modulate chemotaxis by inhibiting the secretion of pro-inflammatory cytokines from macrophages (Patel, Montero-Melendez et al. 2011). For example, the selective MC3R agonist [D-Trp^8]-γ-MSH alleviates the secretion of chemokines CXCL1 and chemokine (C-C motif) ligand 2 (CCL2). This effect is absent in *Mc3r* knockout mice (Leoni, Patel et al. 2008). Similarly, MC3R deficiency increases the expression of interleukin 1β (*Il1β*), interleukin 6 (*Il6*), *inos*, *Ccr4*, *Cxcr3* and *Ccl2* in the

mouse joint samples compared with wildtype (WT) (Patel, Leoni et al. 2010). This evidence shows that MC3R contributes to the anti-inflammatory actions of the melanocortin system.

In addition to MC3R, MC1R mediates the anti-inflammatory actions of melanocortins in macrophages (Catania, Gatti et al. 2004). On the contrary to MC3R, MC1R expression is markedly upregulated upon endotoxin stimulation leading to the suppression of NF-κB-related gene products. These effects are absent in MC1R siRNA knockdown macrophages. (Li, Taylor 2008)

Melanocortins also influence the polarisation of macrophages. Rinne et al. showed that long-term MT-II treatment could induce the polarisation of macrophages into anti-inflammatory M2 type and inhibit the expression of proinflammatory molecules (Rinne, Silvola et al. 2014).

2.2.3.5 Lymphocytes

In humans, 20-30% of the blood leukocytes are lymphocytes. They include B, T and natural killer (NK) cells. Activated B cells, plasma cells, secrete antibodies against the pathogen mediating the humoral immunity. T cells, on the contrary, act locally in the lymph tissue or at the site of inflammation playing a role in the cell-mediated immunity. B and T cells belong to acquired or adaptive immune system, whereas NK cells are a part of the innate immunity. NK cells destroy cells that are infected by viruses and some cancer cells, i.e. those that are unable to express MHC I on their cell surface.

The melanocortin system plays a role in the differentiation of B cells. ACTH and its fragment $ACTH_{1-24}$ maintain the growth of B cells and increase the antibody production (Alvarez-Mon, Kehrl et al. 1985). In addition, α-MSH and its analogue [Nle^4-D-Phe^7]-α-MSH stimulate the growth of B cells (Buggy 1998).

α-MSH also modulates the T cell response. The interaction of α-MSH and MC5R induces the $CD25^+$ and $CD4^+$ T cells to produce anti-inflammatory transforming growth factor β1. (Taylor, Namba 2001)

2.3 The pathogenesis of atherosclerosis

Although atherosclerosis is characterised and known by lipid-rich subendothelial plaques, it is primarily an inflammatory disorder. Several factors, including elevated cholesterol and triglyceride concentrations, initiate the maladaptive immune response in the arteries, which eventually leads to the endothelial dysfunction, recruitment of immune cells, entrapment of lipids and formation of atherosclerotic plaques. The preceding factors are discussed in this chapter.

2.3.1 Endothelial dysfunction

The arteries are composed of different types of layers: the outermost layer, tunica adventitia, is mainly composed of collagen; tunica media, is composed of smooth muscle cells and fibrous tissue; and the innermost layer, tunica intima, supports the cellular monolayer, the endothelium. These three layers are separated by elastic lamina. The disturbance of endothelium is a key step in the development of atherosclerosis (Davignon, Ganz 2004).

The innermost surface of the vasculature, endothelium, is the barrier between the blood material and vascular wall. The endothelium is structured by calveolae vesicles, which enable the transendothelial transport of various substances to the vessel wall. Thus, the endothelium controls the delivery of various substances from the blood into the tissue and *vice versa* (Chistiakov, Orekhov et al. 2015). The transendothelial transport is crucial in both health and disease, because it is needed for angiogenesis, immune response, vascular remodelling and tissue repair (Chistiakov, Orekhov et al. 2015). In healthy vasculature, the permeability of the endothelium is limited. However, upon stressful conditions, such as ischemia or oxidative stress, the endothelial barrier function is impaired. This leads to increased endothelial permeability that is the initiating event of a vascular disease.

The damaged endothelium produces vascular cell-adhesion molecule 1 (VCAM1), which initiates the immune response in the intima (Cybulsky, Gimbrone 1991, Cybulsky, Iiyama et al. 2001). VCAM1 interacts with very late antigen 4 (VLA4) on the

surface of circulating T cells and monocytes, promoting their arrest in the endothelium (Hansson, Libby 2006). Infiltrated immune cells secrete pro-inflammatory chemokines, such as CCL2, that further recruit immune cells into the vascular wall. CCL2 and chemokine (C-C motif) ligand 5 (CCL5) play a crucial role in the development of atherosclerosis because the blocking of their action by knockout models or antagonists alleviates atherogenesis in mice (Boring, Gosling et al. 1998, Gu, Okada et al. 1998). Moreover, another adhesion molecule, ICAM1, is upregulated in the intimal layer of the atherosclerotic plaques, but it has a minor role in the initiation of the disease (Cybulsky, Iiyama et al. 2001). The infiltration of immune cells happens at the first stage of atherosclerosis development; thus these molecules play a crucial role in the disease pathogenesis (Hansson, Libby 2006).

One of the most important functions of the endothelium is to regulate the vascular tone, i.e. the balance between vasoconstriction and vasodilatation. In the damaged endothelium, the endothelium-dependent vasorelaxation is compromised due to a low bioavailability of NO, which shifts the vascular tone towards vasoconstriction (Ludmer, Selwyn et al. 1986). This effect can be observed even before the clinical manifestation of the disease, suggesting that the endothelial dysfunction is an early marker for atherosclerosis (Ludmer, Selwyn et al. 1986).

NO is produced by enzymes called nitric oxide synthases (NOS), which appear in three isoforms: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) nitric oxide synthases (Bredt, Hwang et al. 1990). nNOS is mainly expressed in the nervous system, while eNOS is present in the vascular endothelium, where it induces NO production in response to acetylcholine (ACh) or bradykinin (Fleming, Busse 2003). Most NO is produced by eNOS. The vasodilative influences of NO can be prevented pharmacologically by a competitive NOS inhibitor N^{ω}-Nitro-L-arginine (L-NNA). In addition to vasodilatation, eNOS inhibits platelet aggregation, leukocyte adhesion and proliferation of vascular smooth muscle cells (Forstermann, Closs et al. 1994), which contribute to the development of atherosclerosis. Hence, eNOS is considered as an antiatherogenic enzyme (Fleming, Busse 2003). Both nNOS and eNOS are constantly translated, while iNOS is induced by an inflammatory stimulus, such as LPS, IL1β, TNF-α and oxidative stress, in the vasculature and immune cells (Kleinert, Boissel et al. 2000, Fleming, Busse 2003). *iNOS* expression is under the control of NF-κB (Kleinert,

Boissel et al. 2000); thus, iNOS can be considered as a marker for inflammation. Indeed, iNOS is upregulated in various inflammatory diseases and its expression level correlates with the disease severity (Kröncke, Fehsel et al. 1998). When induced, iNOS promotes the synthesis of vascular endothelial growth factor and collagen that enhance wound healing (Thornton, Schäffer et al. 1998). However, these processes also contribute to the pathogenesis of atherosclerosis.

2.3.2 Lipid oxidation

The main feature of atherosclerosis is subendothelial plaques, which compose of fibrosis, dead cells, inflammatory cells and lipids, including low-density lipoprotein (LDL) (Hansson, Libby 2006). LDLs are lipid carrier complexes that deliver cholesterol in the blood. A component of LDL, apolipoprotein B-100 (ApoB100), interacts with low-density lipoprotein receptor (LDLR) and mediates the intake of LDL into the cell. High lipid concentrations in the blood are associated with atherosclerotic lesion formation and defects in *LDLR* cause high blood cholesterol and heart attacks at young age (Brown, Goldstein 1986). The progression of atherosclerosis is halted when the LDL concentration is below 2 mmol/l and regressed when the concentration is 0.5-1.5 mmol/l. On the grounds of this observation, *Ldlr*$^{-/-}$ mouse fed with high cholesterol diet is often used as a disease model for human atherosclerosis (Ylä-Herttuala, Bentzon et al. 2011). Crossbreeding of *Ldlr*$^{-/-}$ mice with other knockout mice enables to study the mechanisms that affect the development of atherosclerosis (Hansson, Libby 2006).

The circulating LDL is able to accumulate to the proteoglycan structures in the intima in the absence of luminal elastin barrier and in the presence of exposed collagen/proteoglycan network (Kwon, Schroeder et al. 2008). Because there is no lymphatic system in the intima, the removal of LDL particles is very slow. Upon oxidative stress, subendothelial LDL accumulation leads to the oxidation of the lipids. Macrophages recognise and internalise the modified LDL particles via scavenger receptors with high affinity, which further triggers the immune response and the endothelial damage in the vasculature (Weber, Noels 2011). This leads to the subendothelial thickening of the intima, i.e. the formation of early lesions, fatty streaks. The current treatment of atherosclerosis aims at lowering plasma LDL levels which reduces the entrapment of LDL to the intima. However, none of the current treatments

inhibit the LDL oxidation itself. Despite the strong evidence of the role of LDL in the development of atherosclerosis, the LDL modification hypothesis has also been criticised (Steinberg 2009).

2.3.3 Macrophages

The monocyte-derived macrophages are the main component of the plaques (Schaffner, Taylor et al. 1980). The internalisation of native LDL by macrophages is relatively slow, but when LDL is modified, the engulfment of LDL is more rapid (Henriksen, Mahoney et al. 1981). When exposed to modified LDL, endothelial cells and smooth muscle cells in the arteries secrete macrophage-stimulating factor (M-CSF) and granulocyte macrophage-stimulating factor (GM-CSF), which drive the differentiation of monocytes into macrophages (Smith, Trogan et al. 1995). The differentiation amplifies the expression of Toll-like receptors (TLRs) and the scavenger receptors (for example CD36 and CD68) on the cell membrane of macrophages that then internalise the modified LDL (Goldstein, Ho et al. 1979, Kodama, Reddy et al. 1988, Nicholson, Frieda et al. 1995, Ramprasad, Terpstra et al. 1996, Medzhitov, Preston-Hurlburt et al. 1997, Edfeldt, Swedenborg et al. 2002). Excessive lipid accumulation in macrophages eventually leads to the formation of foam cells (Moore, Tabas 2011).

The internalisation of LDL by macrophages protects the endothelial and smooth muscle cells from the damaging effect of oxidised lipids, but it also promotes the production of pro-inflammatory cytokines, such as IL1 that furthers and maintains the inflammation in the vasculature (Hajjar, Haberland 1997). In the late state of atherosclerosis, foam cells may be subjected to necrosis, hereby promoting the lipid core formation of the lesion as the internalised lipids are released to the extracellular space (Viola, Soehnlein 2015).

Macrophages, among other types of cells, secrete matrix metalloproteases (MMPs) as an adaptive response to atheroma formation (Sarén, Welgus et al. 1996, Ylä-Herttuala, Bentzon et al. 2011). The substrates of MMPs include collagen, elastin, gelatine, matrix glycoproteins, and proteoglycans; thus, they degrade extracellular matrix (Newby 2006). MMPs degrade the collagen fibres in the atheroma, which thins the fibrous cap and makes it more susceptible to rupture (Vacek, Rehman et al. 2015). Therefore, the increased MMP secretion correlates with instable lesions (Vacek, Rehman et al. 2015).

Indeed, plaques with a thin cap and lipid-rich necrotic core are thought to be most vulnerable to rupture, and therefore, prone to cause clinical events, such as myocardial infarction (Ylä-Herttuala, Bentzon et al. 2011).

Infiltrated monocytes can develop into various classes of macrophages depending on the environment in the arterial wall, i.e. the exposure to growth factors (GM-CSF, G-CSF), cytokines (TNF-α, interferon γ, IL4, IL10 and IL13), chemokines (CXCL4) and other substances including oxLDL (Viola, Soehnlein 2015). The most thoroughly studied populations are M1 and M2 macrophages, which can be classified as pro-inflammatory and anti-inflammatory macrophages, respectively (Stein, Keshav et al. 1992, Edwards, Zhang et al. 2006). However, the distribution of macrophages into two classes is complex because M1 and M2 macrophages share similar features. It is also unclear whether macrophage polarisation is determined once or if it is a constantly adaptable process depending on the continuously changing environment (Mantovani, Garlanda et al. 2009). The presence of TNF-α and interferon γ favours M1 polarisation, whereas IL4, IL10 and IL13 exposure drives M2 macrophage phenotype (Viola, Soehnlein 2015). M1 macrophages are considered to impair the production of eNOS. Furthermore, pro-inflammatory *iNOS* expression is increased in M1 type macrophages in several inflammatory diseases. *iNOS* expression is under the control of NF-κB. However, anti-inflammatory mediators, such as IL10, suppress NF-κB, and thus, decrease the expression of pro-inflammatory mediators (Wang, Wu et al. 1995, Ckless, van der Vliet et al. 2007). In atherosclerosis, pro-inflammatory M1 macrophages dominate, sustaining the inflammation in the vasculature (Viola, Soehnlein 2015).

Macrophages, along with dendritic cells, can also act as antigen-presenting cells (APCs) that attract and activate T cells. Foam cells that have internalised oxLDL can activate adaptive immunity by processing and presenting a fragment of oxLDL, ApoB, to $CD4^+$ T cells via the interaction of TCR and MHC II. Antigen presentation activates $CD4^+$ T cells to T_H1 cells, which mediate the inflammation by producing cytokines, including IL12, IL18, interferon γ and TNF-α. It has been demonstrated that T_H1 cytokines outnumber in atherosclerosis. Because T_H2 cells inhibit T_H1 response, it has been proposed that atherosclerosis could be alleviated by inhibiting T_H1 response. (Hansson, Libby 2006)

The pathogenesis of atherosclerosis is illustrated in Fig. 8.

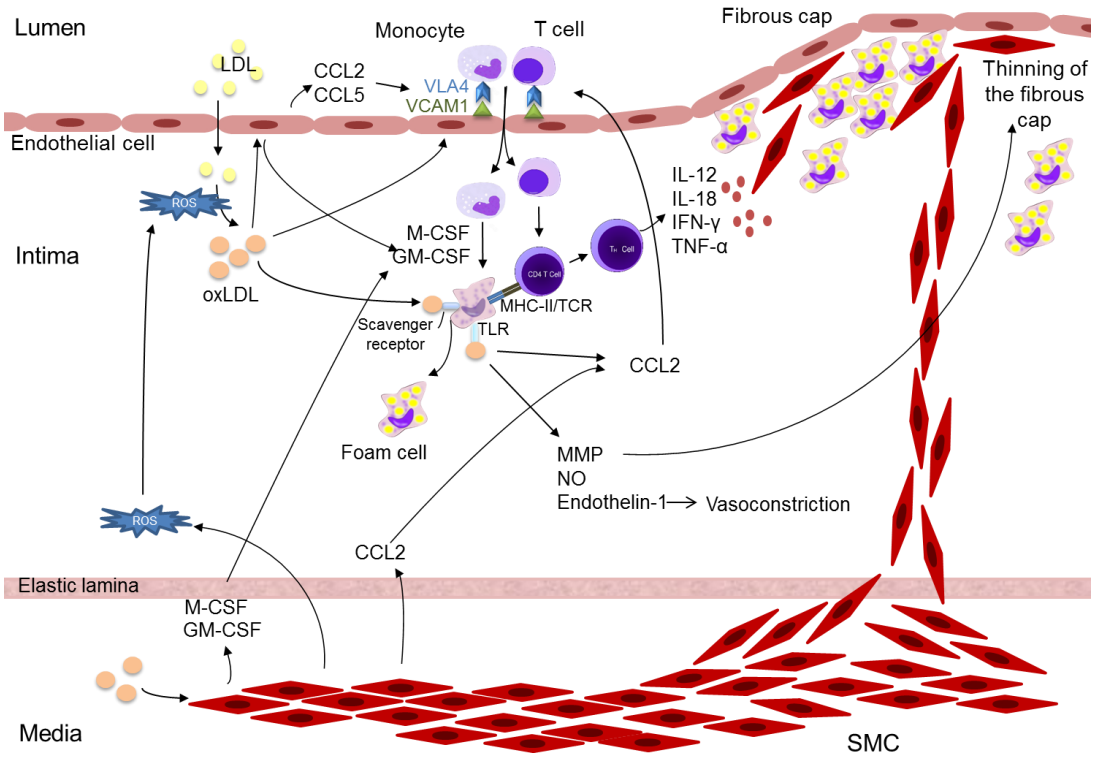

Fig. 8. The scheme of the pathogenesis of atherosclerosis. The inflammatory cascade in the artery is initiated by endothelial damage. Damaged endothelium expresses adhesion molecules (VCAM1) that interact with VLA4 on the surface of circulating monocytes and T cells. Damaged and dysfunctional endothelium permits the infiltration of immune cells as well as LDL into the arterial wall. Infiltrated monocytes differentiate into macrophages that recognise and engulf LDL that has been modified by reactive oxygen species (ROS). Lipid-loaded macrophages turn into foam cells that form the major component of the plaques. In addition, macrophages can present ApoB particles to $CD4^+$ T cells that differentiate to Th_1 cells and secrete pro-inflammatory mediators. oxLDL exposure also recruits smooth muscle cells (SMCs) from the media to the subendothelial space, where SMCs contribute to the plaque formation. As an adaptive response, macrophages and other cells secrete matrix metalloproteinases (MMPs) that digest extracellular matrix in the plaques. Although this process restrains the narrowing of the lumen, it also thins the fibrous cap and makes the plaque more vulnerable to rupture. Figure is modified from Hansson & Libby 2006.

3 AIMS OF THE STUDY

The current study was undertaken to investigate the effects of the trangenic melanocortin OE in Ldlr-deficient mouse model of atherosclerosis. By studying mice fed either regular CRM diet or Western-style diet, we aimed to evaluate the progression

of atherosclerosis in early and advanced phases of the disease, respectively. This study is a continuation of our previous studies indicating that 4-week melanocortin treatment has anti-inflammatory and vasoactive effects in pre-established atherosclerosis. The aims of the present study were:

1) to study whether trangenic α- and γ_3-MSH-OE ameliorates vascular function and mechanical properties of the aorta in atherosclerosis
2) to evaluate whether transgenic α- and γ_3-MSH-OE restrains the aortic inflammation in atherosclerosis
3) to investigate whether transgenic α- and γ_3-MSH-OE limits the plaque formation or alters the plaque characteristics

4 MATERIALS AND METHODS

4.1 Animals

To study the long-term effects of the α- and γ_3-MSH-OE in atherosclerosis, previously generated transgenic mouse model overexpressing α- and γ_3-MSH was crossbred with *Ldlr*$^{-/-}$ mice (Savontaus, Breen et al. 2004). The transgene encodes *N*-terminal POMC, including α- and γ_3-MSH and is under the control of the cytomegalovirus promoter that drives the expression of *N*-terminal POMC in all tissues. However, because prohormone convertases, which post-transcriptionally process *N*-terminal POMC to biologically active melanocortin peptides, are expressed only in certain cell types, α- and γ_3-MSH-OE is limited to these tissues (Benjannet, Rondeau et al. 1991). Thus, in this animal model, α- and γ_3-MSH are processed correctly only in the tissues that normally process melanocortins, including the brain, skin, liver and heart (Savontaus, Breen et al. 2004). In these tissues α- and γ_3-MSH concentrations are elevated two-fold compared to WT controls (Savontaus, Breen et al. 2004).

Transgenic female (n=10) and male (n=17) MSH-OE-Ldlr$^{-/-}$ mice and their *Ldlr*$^{-/-}$ female (n=11) and male (n=20) controls were fed either with regular CRM diet or high-fat, –cholesterol and –sugar Western diet (D12079B, Research Diets Inc.) to accelerate the disease development. Female mice were only fed with Western diet. Until the age of 3 months, all mice were fed regular CRM diet. At the age of 3 months, 18 male and 21

female mice were placed on Western diet while CRM group of 19 male mice continued on the regular diet. The energy content of the Western diet was 4.7 kcal/g and it composed of 17 kcal% protein, 43 kcal% carbohydrate, and 41 kcal% fat where 0.21 kcal% came from cholesterol. During the diet intervention, body weight and food consumption were monitored weekly. Thereafter, the tissues were weighted and collected for further analysis. The duration of the diet was determined on the grounds of previous evidence showing that *Ldlr*$^{-/-}$ mice develop wide atherosclerotic lesions after 4 months on high-fat diet (Silvola, Saraste et al. 2011). All experiments were carried out in homozygous MSH-OE *Ldlr*-deficient (MSH-OE) and in *Ldlr*-deficient (WT) mice. The study protocol is presented in Fig. 9.

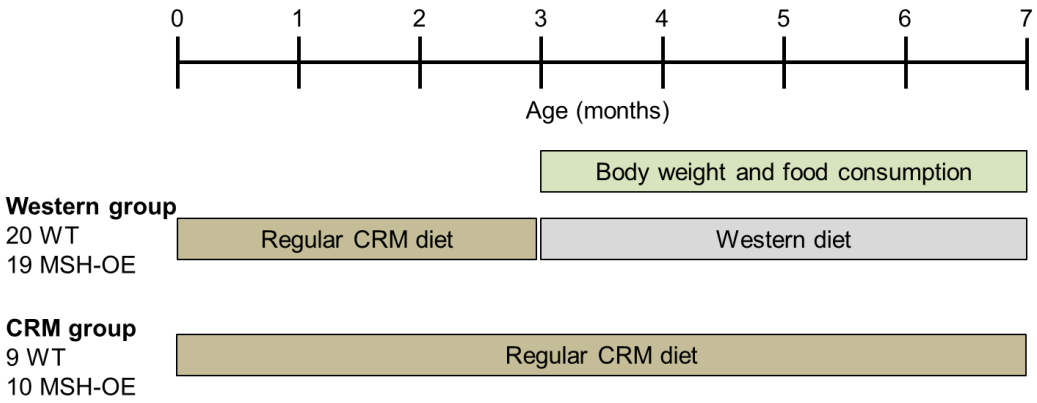

Fig. 9. The study protocol. At the age of 3 months, MSH-OE and WT mice were divided into CRM or Western groups. During 16-week diet-intervention, body weight and food consumption were monitored weekly after which the tissues were collected for further analysis.

The experiments were planned to minimize the number of animals to be used. Euthanasia was carried out via CO_2 asphyxiation. All animal experiments were approved by the Animal Experiment Board in Finland (license number ESAVI-438/04.10.03/2012) and were performed according to European Union Directive 2010/63/EU.

4.2 *Ex vivo* vascular studies

Rapidly after euthanasia, the thoracic aorta from the aortic arch to the diaphragm was placed in ice-cold oxygenated Krebs solution. Dissected 2 mm aortic ring was mounted

in a wire-myograph apparatus (Danish Myograph Technologies, Aarhus, Denmark) by threading it onto two wires (Ø=40 µm) and securing it to two supports. The wire-myograph system allows controlling the circumference of the vessel while recording the force that the two wires place on the aortic ring. After mounting, vessel segment was allowed to equilibrate for at least 20 minutes before normalisation to 100 mmHg physiological tension. (Rinne, Nordlund et al. 2013)

During the functional measurements, the distance between the two wires was kept constant. Isolated aorta was contracted three times with 62 mM KCl to determine the maximal contraction of the vessel. The vessel segments that were unresponsive to potassium were rejected from the results because unresponsiveness to potassium indicates that the vascular smooth muscle integrity is damaged. α_1-adrenergic receptor-mediated vasoconstrictor response was determined by cumulative doses of phenylephrine. Vessel was precontracted with 1 mM prostaglandin $F_{2\alpha}$ to obtain 50-80% of the maximal reference contraction to KCl for the monitoring and quantification of ACh-induced endothelium-dependent vasodilatation. Endothelium-independent relaxation was studied by adding cumulative concentrations of sodium nitroprusside (SNP), that acts as a vasodilator by releasing NO, to precontracted vessels. The contribution of NO to endothelium-dependent vasodilatation was determined by incubating the aortic ring with 100 µM L-NNA for 30 minutes before contracting the vessels with phenylephrine, and subsequently relaxing them with ACh. L-NNA is a competitive NOS inhibitor, and thus, prohibits the formation of NO. Vessel baths were washed at least three times with fresh, warm, and oxygenated Krebs solution between the different functional experiments. Krebs solution is a physiological salt solution composed of 119 mM NaCl, 25 mM $NaHCO_3$, 5.5 mM glucose, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$ and 2.5 mM $CaCl_2 \cdot 2H_2O$.

After functional experiments, aortic segment was equilibrated 30 minutes and subsequently studied for mechanical properties by a graded passive distension procedure. The two wires were brought together so that no tension was placed in the ring preparation and the micrometre was zeroed (L_0). Then, the distance between the wires was increased stepwise by 20 µm every 60 seconds, until the micrometre reading reached 100 µm, and subsequently by 10 µm every 60 seconds until 30-40 mN wall force was reached. The wall force and micrometre reading were recorded at each step,

and the related wall stress (mN/mm) was calculated and plotted against the resulting strain ($\Delta L/L_0$). (Van Herck, De Meyer et al. 2009, Hadjinikolaou, Kotidis et al. 2004, Rinne, Ahola-Olli et al. 2015)

During the functional and mechanical experiments, vessel segments were kept in oxygenated Krebs solution (95% O_2 and 5% CO_2) and heated to 37°C. Krebs solution in the myograph wells was discarded at least every 20 minutes and fresh, warm and oxygenated Krebs solution was provided to maintain the physiological properties of the vessels. Chart5 and PowerLab (ADI Instruments, Colorado Springs, CO) were used for the data recording and analysis.

4.3 Plaque characterisation

4.3.1 En face stainings

The adventitia around the aortic arch was removed and the aortic arch was dissected. Aortic arch samples were fixed in 10% formalin for 24 hours and stored in phosphate-buffered saline (PBS) at 4°C until staining. Any remaining adventitia from the aortic arch was removed before staining. The fixed aortic arch was dissected longitudinally open from the heart to the left subclavian artery and pinned flat intima upward.

En face preparations of the aortic arch were stained with Sudan IV for atherosclerotic plaque quantification. 70% ethanol was added to the dish for 5 minutes. Filtered 0.5% (wt/vol) Sudan IV solution was applied for 6 minutes. Sudan IV (Sigma-Aldrich) was dissolved equally in acetone and 70% ethanol. Aortic arches were destained with 80% ethanol for 3 minutes and washed with PBS. The en face aortae were mounted on a glass plate under a coverslip using PBS. For quantitative analysis, images of the stained tissues were captured using Zeiss Stemi 2000-C stereomicroscope and Pixelink software. The intimal area was limited using image manipulation programme (GIMP 2.8, GNU Image Manipulation Program). The atherosclerotic plaque area of the total intimal area was determined using automated image analysis software (ImageJ, Fiji, National Institutes of Health, Bethesda, Maryland, USA) with colour deconvolution plug-in (Ruifrok, Johnston 2001, Chen, Quan et al. 2010).

4.3.2 Immunohistochemical and histological stainings

The aortic roots were embedded in Tissue-Tek O.C.T. (Sakura), frozen in isopentane on dry ice and stored at -70°C until further use. Transverse sections of aortic root (8 µm) were cut and stained with Oil Red O, Masson's Trichrome, and Mac-3 and iNOS antibodies for the evaluation of lipid accumulation, collagen deposition, macrophage density and polarisation, respectively (Rinne, Silvola et al. 2014). For iNOS immunohistochemistry, the primary antibody concentration and fixation method were first optimised using three different primary antibody concentrations (1:50, 1:100 and 1:200) and either methanol or acetone fixation and the results were evaluated under microscope. Finally, 1:100 primary antibody dilution and cold acetone were chosen for the staining. Aortic sections were fixed with cold acetone and staining was performed according to Abcam immunohistochemistry protocol in Scientific Shandon Sequenza Immunostaining Center (Thermo Fisher). Endogeneous peroxidase activity was suppressed with 1% H_2O_2 and the background staining was minimised by 1% bovine serum albumin (Sigma-Aldrich). Primary iNOS antibody (ab15323, Abcam) was applied and incubated for 18 hours in 4°C to ensure sufficient antibody binding. Using indirect immunohistochemistry method, the primary antibody was visualised by horseradish peroxidase-conjugated secondary antibody (ABC kit, Vector Laboratories) (Hsu, Raine et al. 1981). Finally, brown colour was developed with 3,3'-diaminobenzidine tetrahydrochloride (D5637, Lot 60K0820, Sigma-Aldrich) and nuclei were counterstained with hematoxylin. The stained sections of the aortic roots were scanned using Pannoramic 250 digital slide scanner (3DHISTECH Ltd.) and the areas of intima and media were delimited using image editing software (GIMP 2.8, GNU Image Manipulation Program). To characterise the plaque features, the areas of intima and media were analysed by using an automated image analysis software (ImageJ, Fiji, National Institutes of Health, Bethesda, Maryland, USA) with colour deconvolution plug-in, which separates brown (macrophage), red (lipid) and blue (collagen) components (Ruifrok, Johnston 2001).

4.4 Quantitative reverse-transcription polymerase chain reaction (RT-qPCR)

The total RNA was isolated from the aorta by phenol/guanidine-based extraction (QIAzol Lysis Reagent, Qiagen). RNA quality and concentration were measured by spectrophotometer (BioSpec-nano, Shimadzu) and the RNA was reverse-transcribed to complementary DNA (Applied Biosystems) using 2729 Thermal Cycler (Applied Biosystems). RT-qPCR was carried out by 7300 Real Time PCR System (Applied Biosystems) and SYBR Green (KAPA Biosystems) was used to detect PCR products. Each sample was run in duplicate. Primer sequences for mouse genes are shown in Table 2. Target gene mRNA expression levels were normalised to the endogenous ribosomal S29 expression. The average ΔCt value of CRM WT samples served as a calibrator.

Table 2. RT-qPCR primers for mouse genes.

Gene name Accession number	**5'-3' primer sequence**
Il1β NM_008361.3	Forward: CCACCTTTTGACAGTGATGA Reverse: GAAGCTCTTGTTGATGTGCT
Il6 NM_031168.1	Forward: ACAACCACGGCCTTCCCTACTT Reverse: CACGATTTCCCAGAGAACATGTG
Ccl2 NM_011333.3	Forward: AGGTCCCTGTCATGCTTCTG Reverse: AAGGCATCACAGTCCGAGTC
Ccl5 NM_013653.3	Forward: TGCTGCTTTGCCTACCTCTC Reverse: TTGGCACACACTTGGCGGTT
Nos2 NM_ 001313921.1	Forward: CCTGCACGTCTTTGACGCTCGG Reverse: TTTGCCGTCACTCCGCTGGG
Nos3 NM_008713.4	Forward: GGCACTGCTGAGCCGAGTGG Reverse: GAGCCTGCCGCAGCGTACAT
S29 NM_009093.2	Forward: ATGGGTCACCAGCAGCTCTA Reverse: AGCCTATGTCCTTCGCGTACT

4.5 Statistical analyses

Statistical differences were calculated by 2-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* tests, when multiple groups were compared or by unpaired two-tailed t test, when only two groups were compared. Concentration-response curves in *ex vivo* wire-myograph were analysed by comparing maximal effect (E_{max}) and half maximal effective concentration (EC_{50}) values using the extra sum-of-squares F test. All statistical analyses were performed using GraphPad Prism 5.0 and 6.0. P values of less than 0.05 were considered statistically significant. All data are presented as mean±standard error of the mean (SEM).

5 RESULTS

5.1 Body and organ weights

Apart from the regulation of cardiovascular and immune responses, the melanocortin system regulates several physiological functions, including energy homeostasis. Hence, we monitored the body weight development during diet-intervention and measured the weight of heart, liver and white adipose tissue (WAT) pads in the end of the study (Tables 2 and 3).

Table 2. Organ weights of male mice. Values are mean (g)±SEM.

		CRM		Western	
		WT	MSH-OE	WT	MSH-OE
	n	9	10	9	9
Body weight		34,9±1,2	34,5±0,75	38,4±2,4	40,6±1,9
Heart		0,169±0,005	0,165±0,005	0,162±0,004	0,153±0,007
Liver		1,758±0,054	1,681±0,052	2,485±0,144	2,361±0,179
Epididymal WAT		0,545±0,072	0,469±0,064	0,913±0,107	0,957±0,074
Retroperitoneal WAT		0,349±0,050	0,255±0,040	0,481±0,055	0,501±0,051

Table 3. Organ weights of female mice. Values are mean (g)±SEM.

		Western	
		WT	MSH-OE
	n	11	10
Body weight		27,1±1,3	25,5±0,4
Heart		0,113±0,003	0,109±0,002
Liver		1,425±0,075	1,403±0,060
Gonadal WAT		0,505±0,065	0,357±0,032
Retroperitoneal WAT		0,239±0,041	0,203±0,018

Body weight was monitored weekly during 16-week diet-intervention, while α- and γ_3-MSH-OE had no effect on the body weight development in either of the diet groups. In females, α- and γ_3-MSH-OE tended to limit the body weight development; however, the difference was statistically nonsignificant (P=0.07) (Fig. 10).

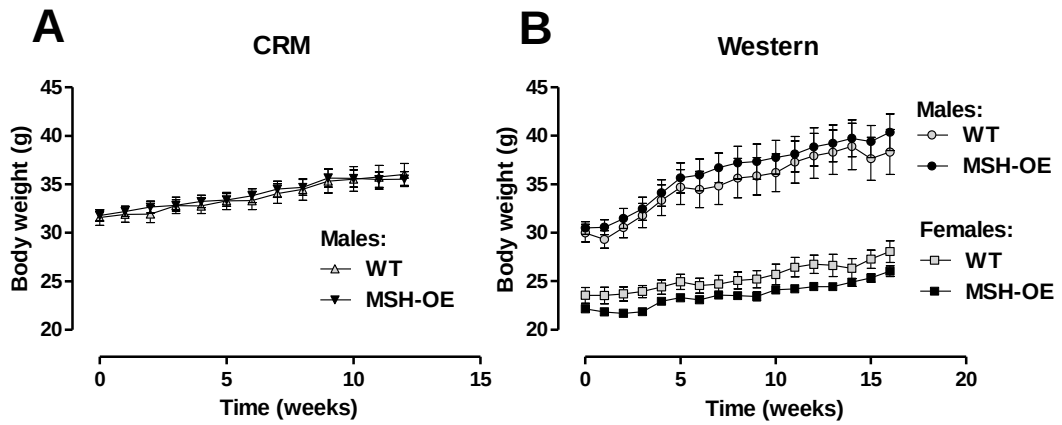

Fig. 10. Body weight development in WT and MSH-OE mice on CRM and Western diet. α- and γ_3-MSH-OE tended to decrease the Western-diet induced body weight development in female mice. Analysis was performed with 2-way ANOVA followed by Bonferroni *post hoc* tests. Data are mean±SEM, n=9-11 per group.

Western diet significantly increased the epididymal and retroperitoneal WAT weights in males ($P<0.0001$ and $P=0.0005$, respectively). There were no differences between the epididymal/gonadal or retroperitoneal WAT weights between the genotypes at the end of the study; however, in females, α- and γ_3-MSH-OE tended to decrease the gonadal WAT pad weight ($P=0.06$). (Fig. 11).

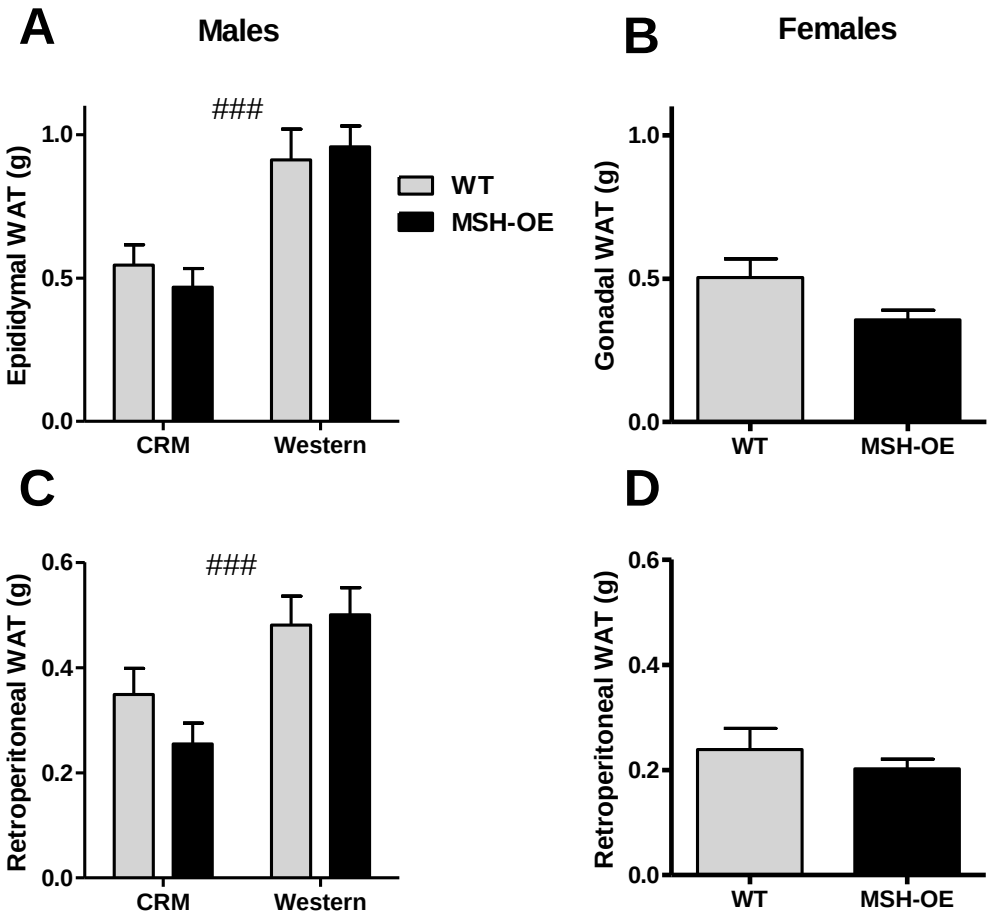

Fig. 11. WAT pad weights of WT and MSH-OE mice in the end of the study. A-B, Epididymal and gonadal WAT weights of male and female mice. **C-D**, Retroperitoneal WAT weights of male and female mice. In male mice, Western diet increased the epididymal and retroperitoneal WAT weights (### P<0.0001 for the diet effect). In females, α- and γ_3-MSH-OE tended to decrease the gonadal WAT weight. Analysis was performed with 2-way ANOVA followed by Bonferroni *post hoc* tests (males) or with unpaired two-tailed t test (females). Data are mean±SEM, n=8-11 per group.

At the end of the study, α- and γ_3-MSH-OE had no effect on body weight in either males or females. However, Western diet significantly increased the body weight in males (P=0.006, Fig. 12A-B). Similarly, Western diet increased the relative liver weight in males (P<0.0001). α- and γ_3-MSH-OE significantly decreased the relative weight of liver in males (P=0.03), while female MSH-OE mice had increased liver weights in the end of the study (P=0.047, Fig. 12C-D). Genotype had no effect on heart to body weight ratio in male or female mice. The diet, however, decreased the heart/body weight ratio in male mice on Western diet (P=0.0004, Fig. 12E-F).

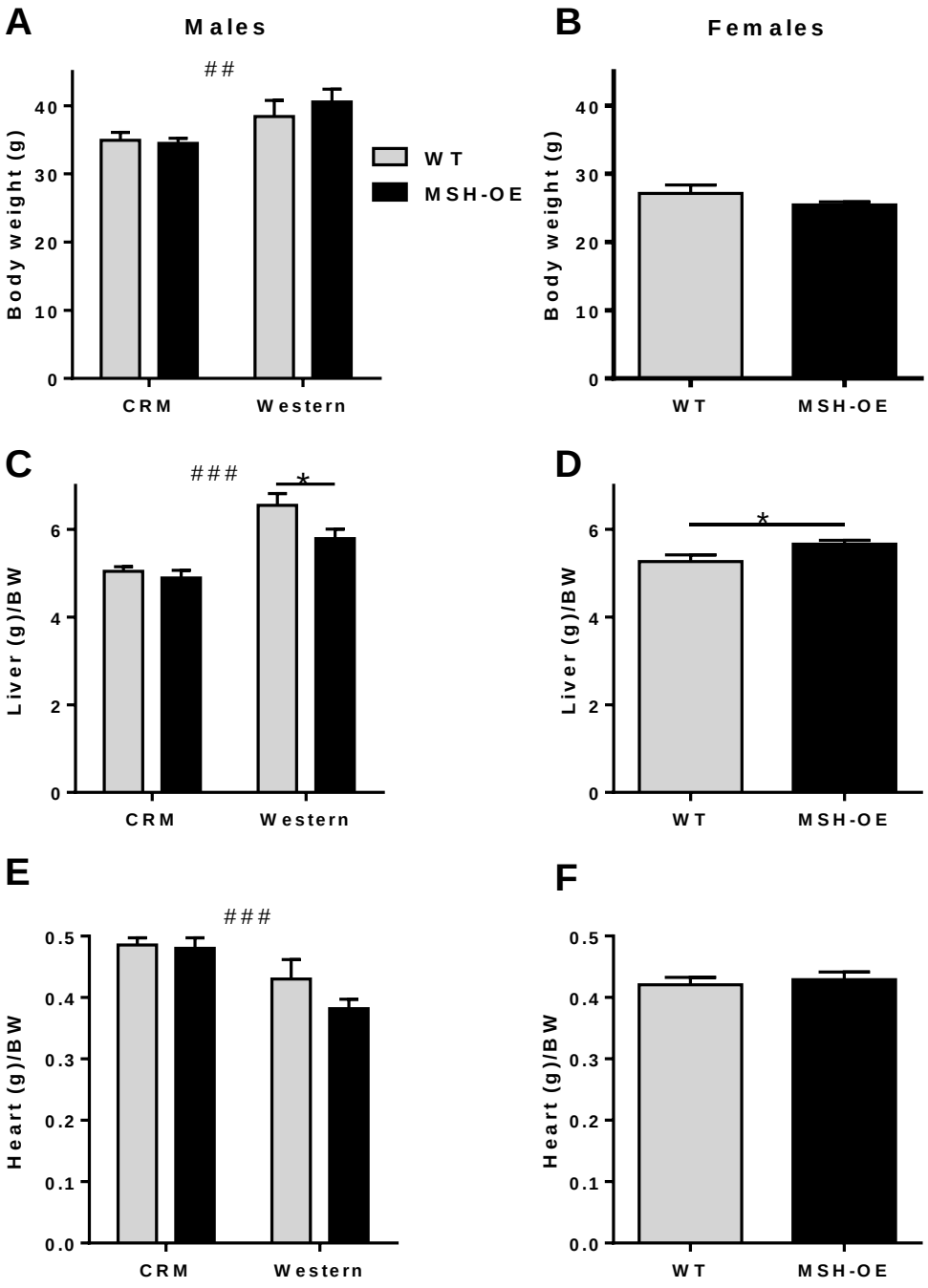

Fig. 12. Body weight and relative weights of liver and heart in the end of the study. **A-B**, Western diet increased the body weight in male mice (## P<0.005 for the diet effect). **C-D**, Male mice on Western diet showed increased liver weight in relation to body weight (### P<0.0001 for the diet effect). On Western diet, male MSH-OE mice had smaller livers than their WT controls (* P<0.05 for the genotype effect). Female mice had increased liver weights. **E-F**, Heart/body weight ratio was smaller in male mice on Western than on regular CRM diet. Analysis was performed with 2-way ANOVA followed by Bonferroni *post hoc* tests (males) or with unpaired two-tailed t test (females). Data are mean±SEM, n=8-11 per group.

5.2 *Ex vivo* vascular studies

To test the hypothesis that α- and γ_3-MSH-OE alleviates vascular dysfunction in atherosclerosis, the functional and mechanical properties of the isolated aortae were examined by wire-myograph.

The isolated aortae were stimulated three times with 62 mM potassium solution. The maximal potassium-evoked constriction was observed on the third, or sometimes on the second, potassium stimulation. In the Western group, the maximal potassium-evoked contractions were weaker than those of regular CRM diet group (P=0.04 for the diet effect, Fig. 13A).

Contractile-responses were also studied using cumulative doses of phenylephrine. The contraction response in the aortae of WT mice compared to the aortae of MSH-OE mice on regular diet was increased (P<0.01, Fig. 13B). Thus, α- and γ_3-MSH-OE restrained the contractile effect of phenylephrine. The α- and γ_3-MSH-OE resisted the contractile responses only in mice placed on CRM, but not on Western, diet (P=0.0015, Fig. 13B-C).

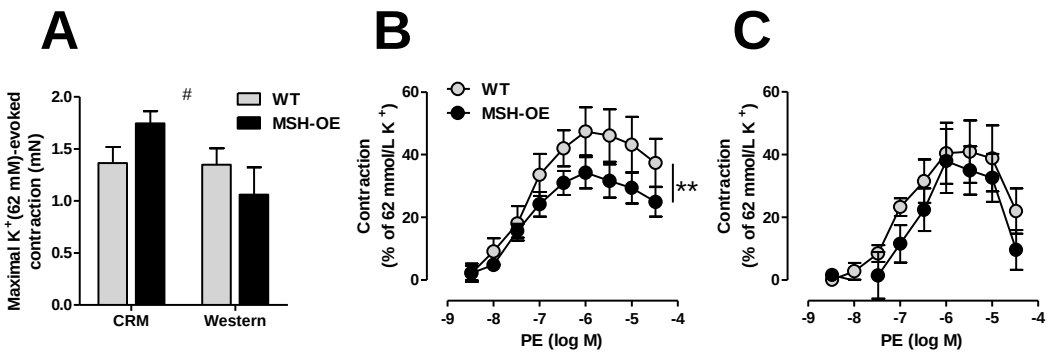

Fig. 13. Contraction responses to potassium and phenylephrine in the isolated aortae of WT and transgenic MSH-OE mice. A, Maximal contraction evoked by 62 mM K^+. On Western diet, the maximal potassium-evoked contractions were weaker than on CRM diet (# P<0.05 for the diet effect). **B-C**, Concentration–response curves for phenylephrine (PE)–induced contractions with regular CRM and Western diet. WT mice had increased contraction responses compared to MSH-OE mice on CRM diet (** P<0.01 for the genotype effect). Analysis was performed by 2-way ANOVA followed by Bonferroni *post hoc* tests and extra sum-of-squares F test. Data are mean±SEM, n=6-10 per group.

After evaluating contractile responses, endothelium-dependent vasorelaxation responses of the aortae were studied. The α- and γ_3-MSH-OE significantly improved the vasorelaxation responses in mice on regular diet ($P<0.0001$ for $logEC_{50}$). There was no improvement in mice placed on Western diet (Fig. 14B). The contribution of NO to this ACh-evoked vasodilatation was studied by adding NOS inhibitor L-NNA to the organ well. Subsequently, the cumulative concentration series of ACh were performed. Now, when the aortae were pretreated with L-NNA and contracted again, the relaxation responses were markedly blunted (Fig. 14C-D).

To evaluate the endothelium-independent vasodilatation responses, NO donor SNP was added to the organ bath. SNP induced a profound relaxation of the aortae in both genotypes (Fig. 14E-F). However, the endothelium-independent responses were similar between the genotypes, suggesting that the genotype effect on vasodilatory responses is limited to endothelium-dependent mechanisms.

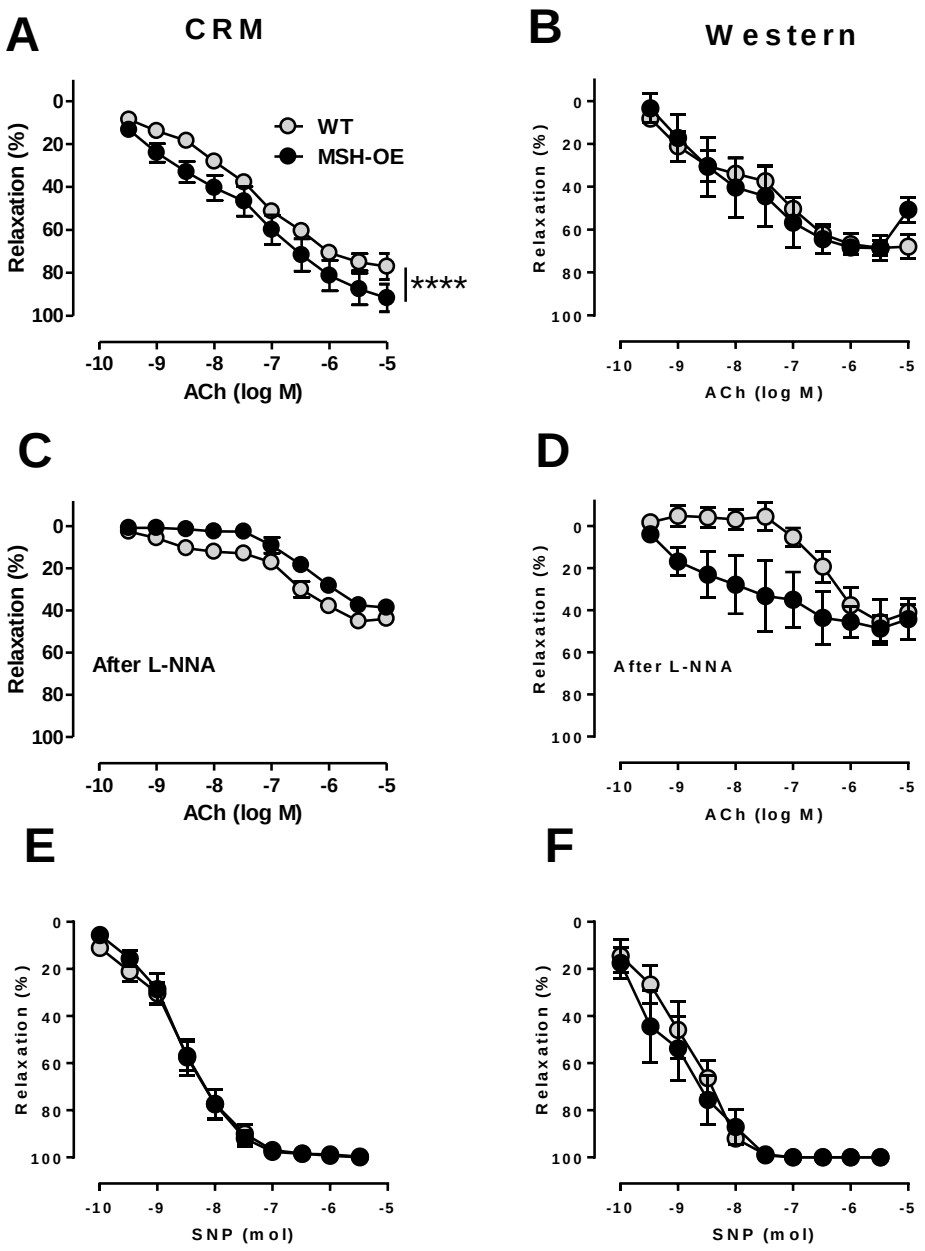

Fig. 14. Vasorelaxation responses of the aortae in WT and MSH-OE mice on regular CRM or Western diet. A-D, Endothelium-dependent relaxations to ACh before and after the addition of L-NNA. **E-F**, Endothelium-independent relaxation response to SNP. α- and γ_3-MSH-OE improved the endothelium-dependent vasorelaxation responses on CRM diet (**** P<0.0001 for genotype effect). The statistical analyses were performed using extra sum-of-squares F test. Data are mean±SEM, n=5-12 per group in each graph.

To characterise the distensibility of the arteries, the isolated aortae were placed on mechanical strain and wall stress-strain curves were obtained. There were no

statistically significant differences in the stress-strain curves between the groups (Fig. 15).

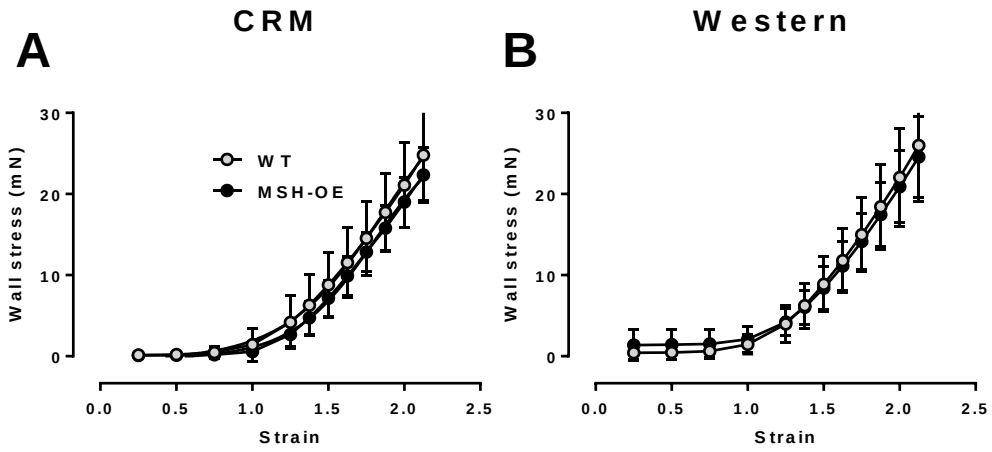

Fig. 15. The stress-strain curves of the aortae of WT and MSH-OE male mice on CRM and Western diets. There was no statistically significant difference in arterial stiffness between MSH-OE and WT mice on regular or Western diet. The statistical analyses were performed using extra sum-of-squares F test. Values are mean±SEM, n=10-14 per group.

5.3 Plaque characterisation

The atherosclerotic plaques in the aortic arch and aortic root were characterised by en face tissue, immunohistochemical and histological stainings.

5.3.1 En face stainings of the aortic arch

To determine intimal plaque deposition in the aortic arch, en face aortic arches were stained with Sudan IV. In male mice, the diet significantly increased the plaque accumulation in the intima ($P<0.0001$ for the diet effect). On the Western diet, MSH-OE mice displayed significantly less intimal plaque accumulation in both males and females compared to WT mice ($P=0.03$ and $P=0.02$, for genotype effect, respectively) (Fig. 16).

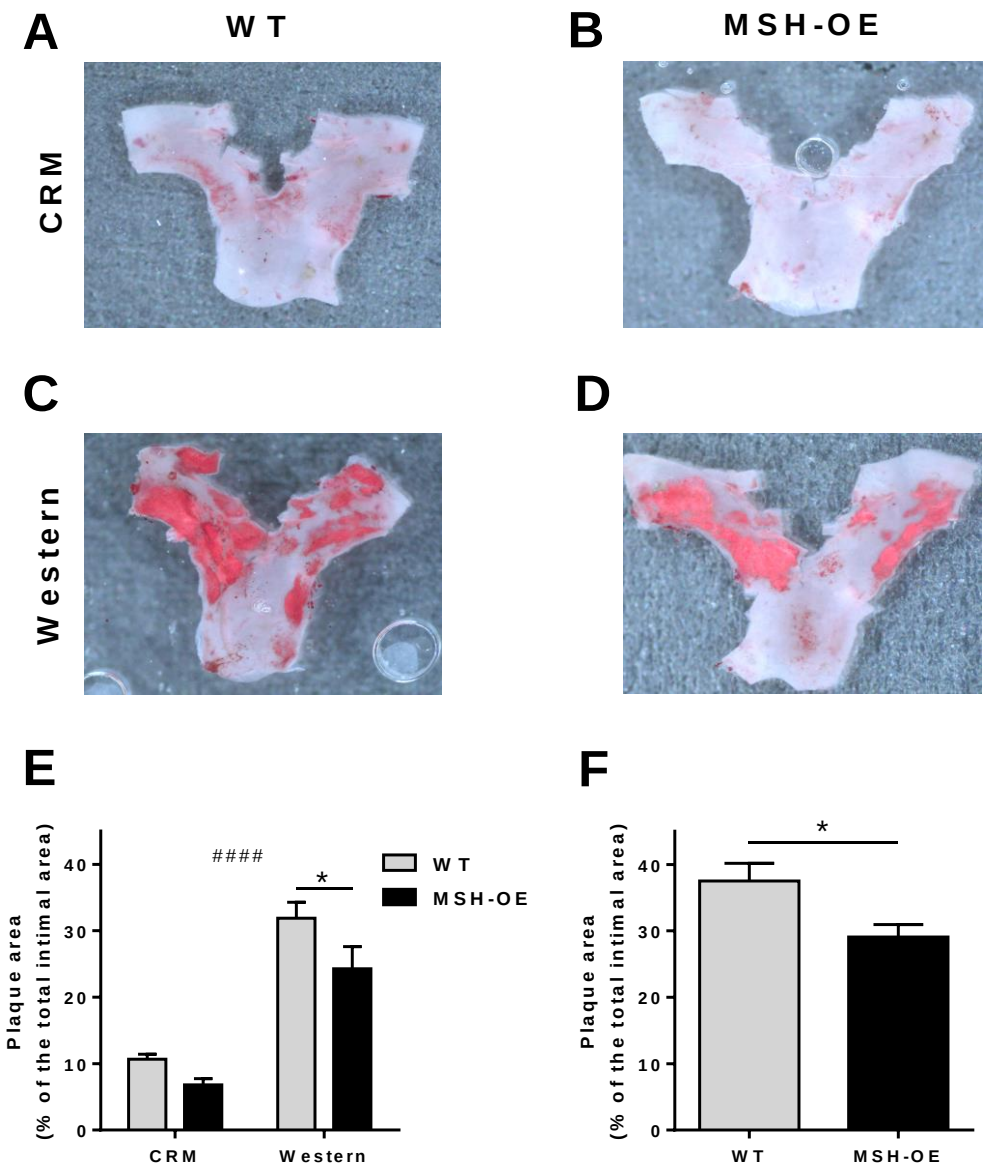

Fig. 16. Plaque area in the aortic arch represented as Sudan IV -stained intimal area. A-D, Representative Sudan IV -stained en face preparations of the aortic arch of WT and MSH-OE mice on CRM or Western diet. **E**, The intimal plaque area was smaller in MSH-OE male mice compared to WT mice on Western diet (* $P<0.05$ for the genotype effect). In males, Western diet significantly increased the plaque development (#### $P<0.0001$ for the diet effect). **F**, In females, α- and γ_3-MSH-OE decreased the plaque development in the aortic arch. Statistical differences were analysed by 2-way ANOVA followed by Bonferroni *post hoc* tests (males) or unpaired t test (females). Values are mean±SEM, n=9-10 per group.

5.3.2 Histological stainings

5.3.2.1 Oil Red O

The absolute lesion area was calculated from Oil Red O stained aortic roots to evaluate the plaque size. Although the genotype had no effect on the plaque formation, the diet significantly increased the plaque size ($P<0.0001$) (Fig. 17).

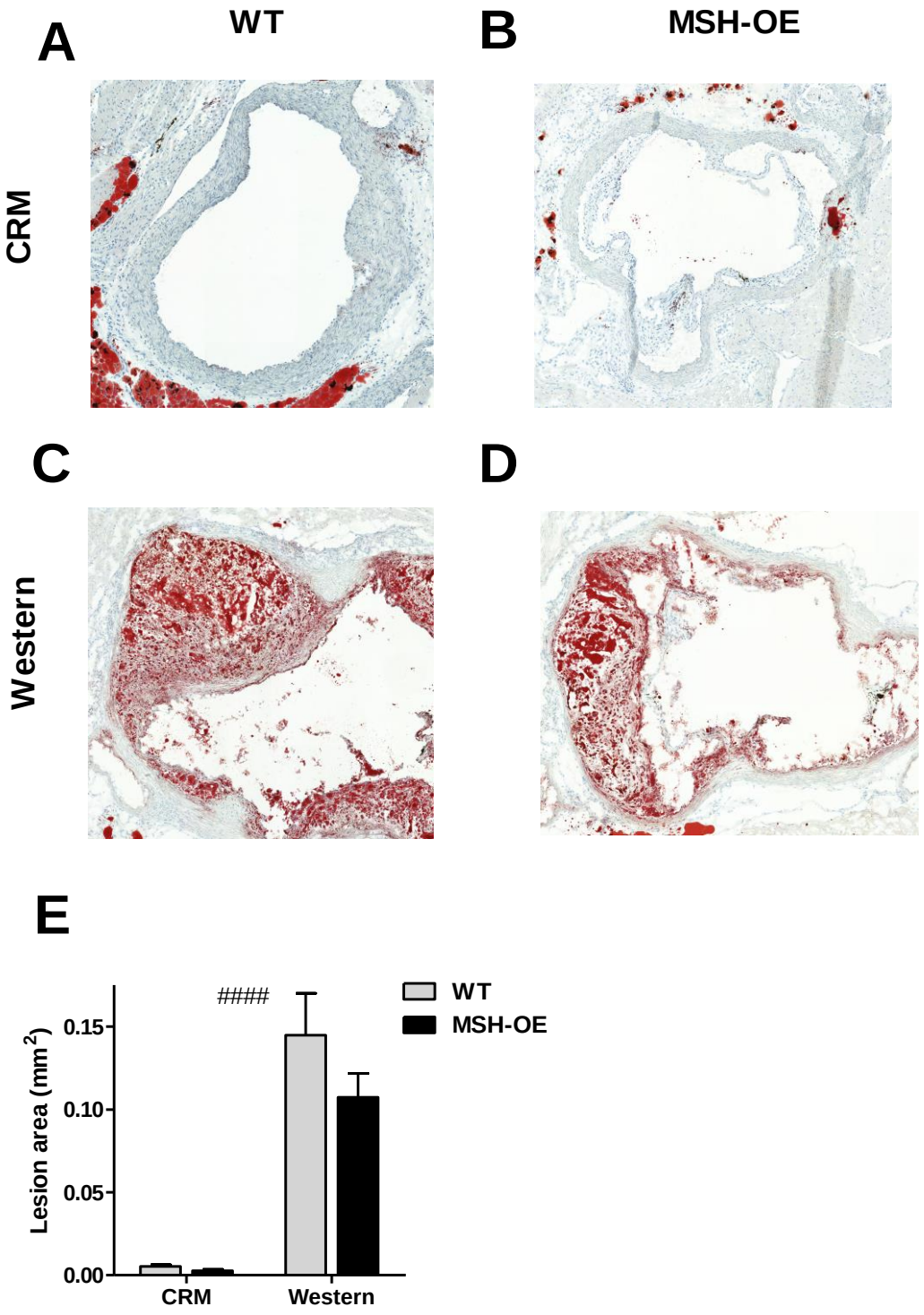

Fig. 17. The absolute lesion area (mm^2) represented as accumulated lipids in the intima in WT and MSH-OE mice on regular CRM or Western diet. A-D, Representative Oil Red O stained sections of aortic root of WT and MSH-OE mice on CRM or Western diet. The diet significantly increased the absolute lesion area (#### P<0.0001 for diet effect). Analysis was performed with 2-way ANOVA followed by Bonferroni *post hoc* tests. Values are mean±SEM, n=7-10 per group.

5.3.2.2 Masson's Trichrome

Masson's Trichrome stained aortic sections were analysed for the evaluation of collagen deposition. Collagen deposition did not differ statistically between the genotypes in males on Western diet (Fig. 18).

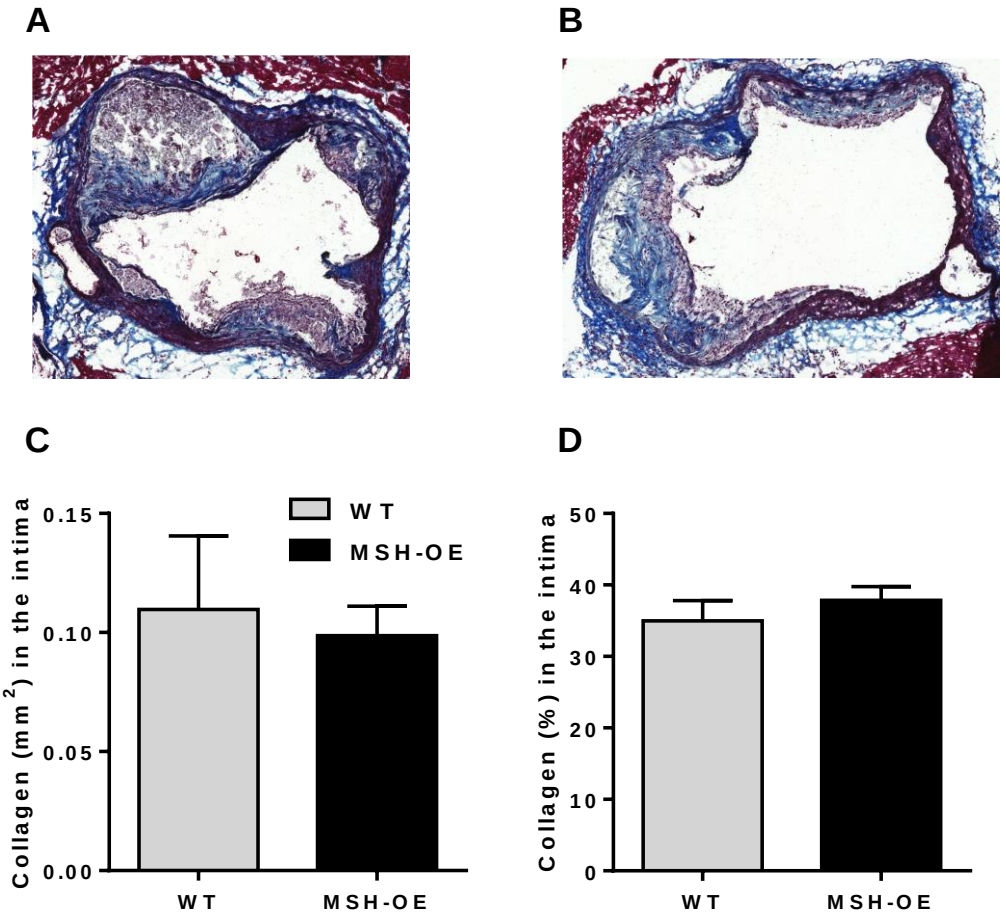

Fig. 18. The absolute (mm^2) and proportional collagen area represented as Masson's Trichrome stained intimal area in WT and MSH-OE mice on Western diet. A-B, Representative Masson's Trichrome stained sections of aortic root of WT and MSH-OE mice on Western diet. **C-D**, The collagen depositions between the genotypes did not differ statistically significantly. Analyses were performed using unpaired t test. Values are mean±SEM, n=6-9 per group.

5.3.3 Immunohistochemical stainings

5.3.3.1 Mac-3

Immunohistochemical Mac-3 stainings of the aortic root were analysed to quantify the macrophage density in the aortic root. There was no significant genotype effect on the proportion of macrophages (Fig. 18B) or the absolute macrophage count in the intima in males on Western diet (Fig. 18C).

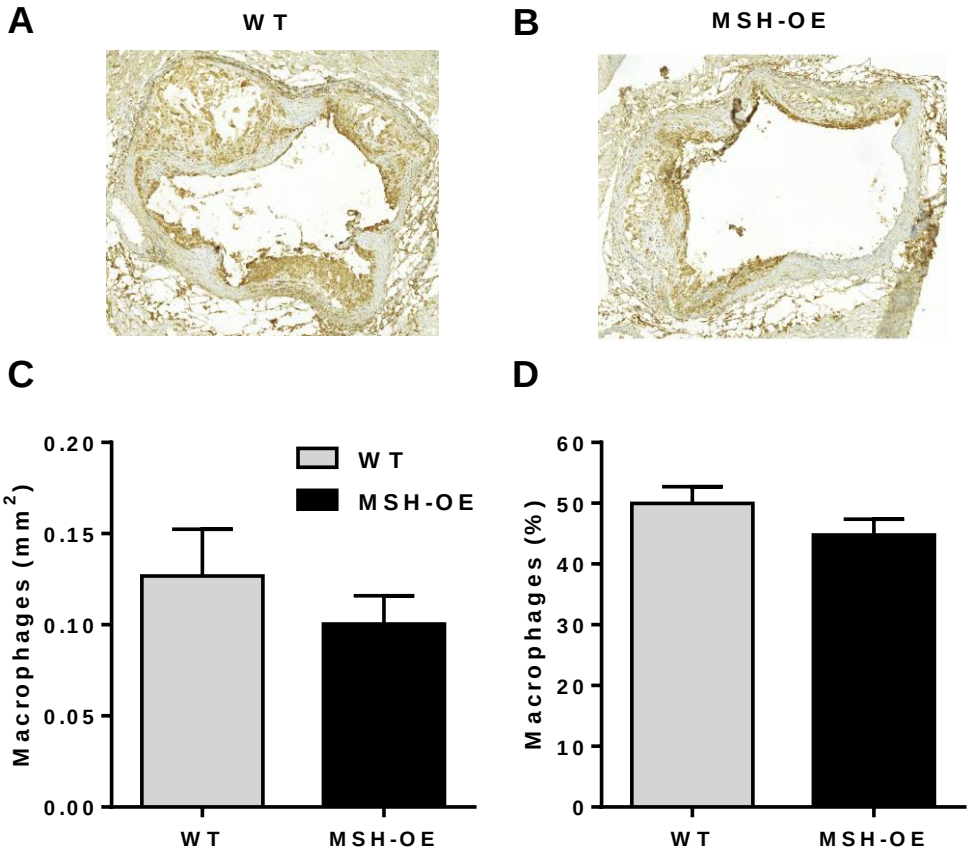

Fig. 18. The absolute (mm^2) and proportional Mac-3-positive area of the intima in WT and MSH-OE mice on Western diet. A-B, Representative Mac-3-stained sections of aortic root of WT and MSH-OE mice on Western diet. **C-D**, The macrophage density and the absolute macrophage count means did not differ statistically significantly between two genotypes. Analysis was performed using unpaired t test. Values are mean±SEM, n=7-9 per group.

5.3.3.2 iNOS

Immunohistochemical iNOS stainings of the aortic root were performed for the characterisation of the macrophage phenotype. The proportion of iNOS-positive area in the intima was smaller in MSH-OE mice, indicating a decreased M1 macrophage deposition (Fig. 18).

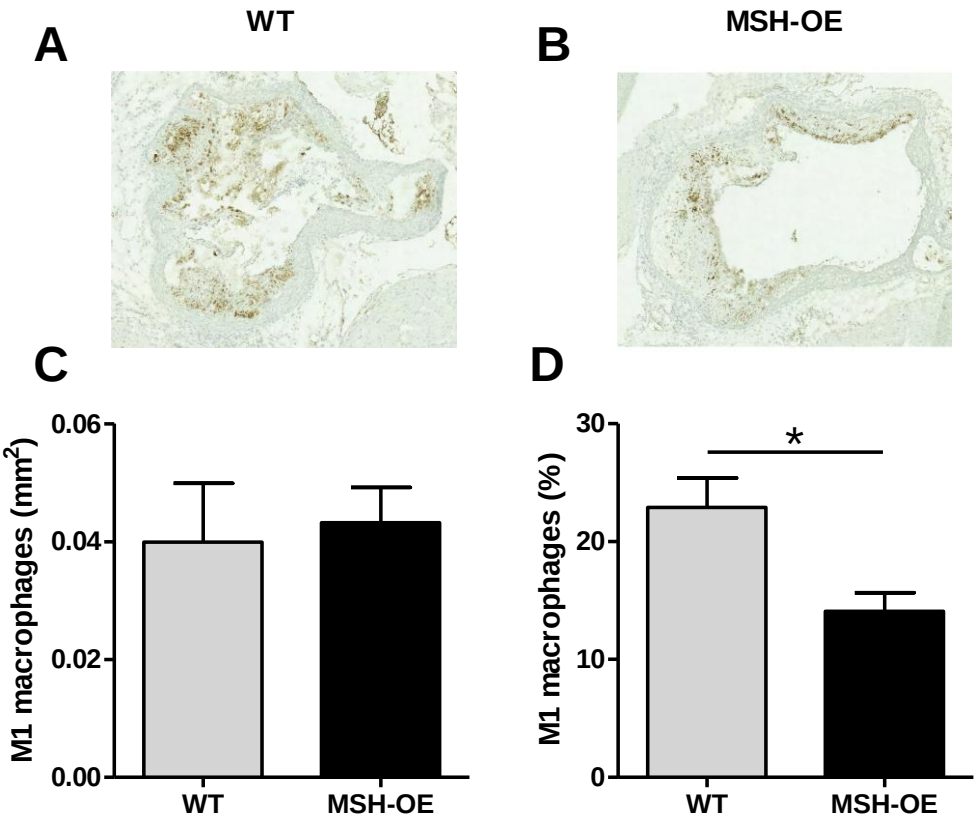

Fig. 18. The absolute (mm^2) and proportional iNOS-positive area in the intima in WT and MSH-OE mice on Western diet. A-B, Representative iNOS-stained sections of aortic root of WT and MSH-OE mice on Western diet. **D**, The proportion of iNOS-stained intimal area was smaller in MSH-OE mice compared with WT mice (* $P<0.05$ for genotype effect). Analysis was performed using unpaired t test. Values are mean±SEM, n=4-9 per group.

5.4 RT-qPCR

Total RNA from the thoracic aortae of WT and MSH-OE mice was extracted and used for the analysis of mRNA expression levels of *Il1β*, *Il6*, *Ccl2*, *Ccl5*, *inos* and *enos* by RT-qPCR.

The mean expression levels of *Il6*, *Ccl2* and *Ccl5* were statistically significantly lower in MSH-OE mice vs. WT mice on Western diet (P=0.04, P=0.003 and P<0.0001, respectively) (Fig. 19B-D). There was no genotype effect of these cytokines on regular diet. The Western diet significantly increased the expression level of *Ccl2* and *Ccl5* (P=0.003 and P<0.0001 for diet effect, respectively); however, the diet effect on *Il6* expression did not quite reach the statistical significance (P=0.07 for the diet effect). α- and γ_3-MSH-OE, thus, seemed to alleviate the diet-induced increase in *Ccl2* and *Ccl5* expression. There was no difference in *inos* or *enos* expressions between the genotypes. However, the Western diet increased *inos* (P=0.008), but not *enos*, expression.

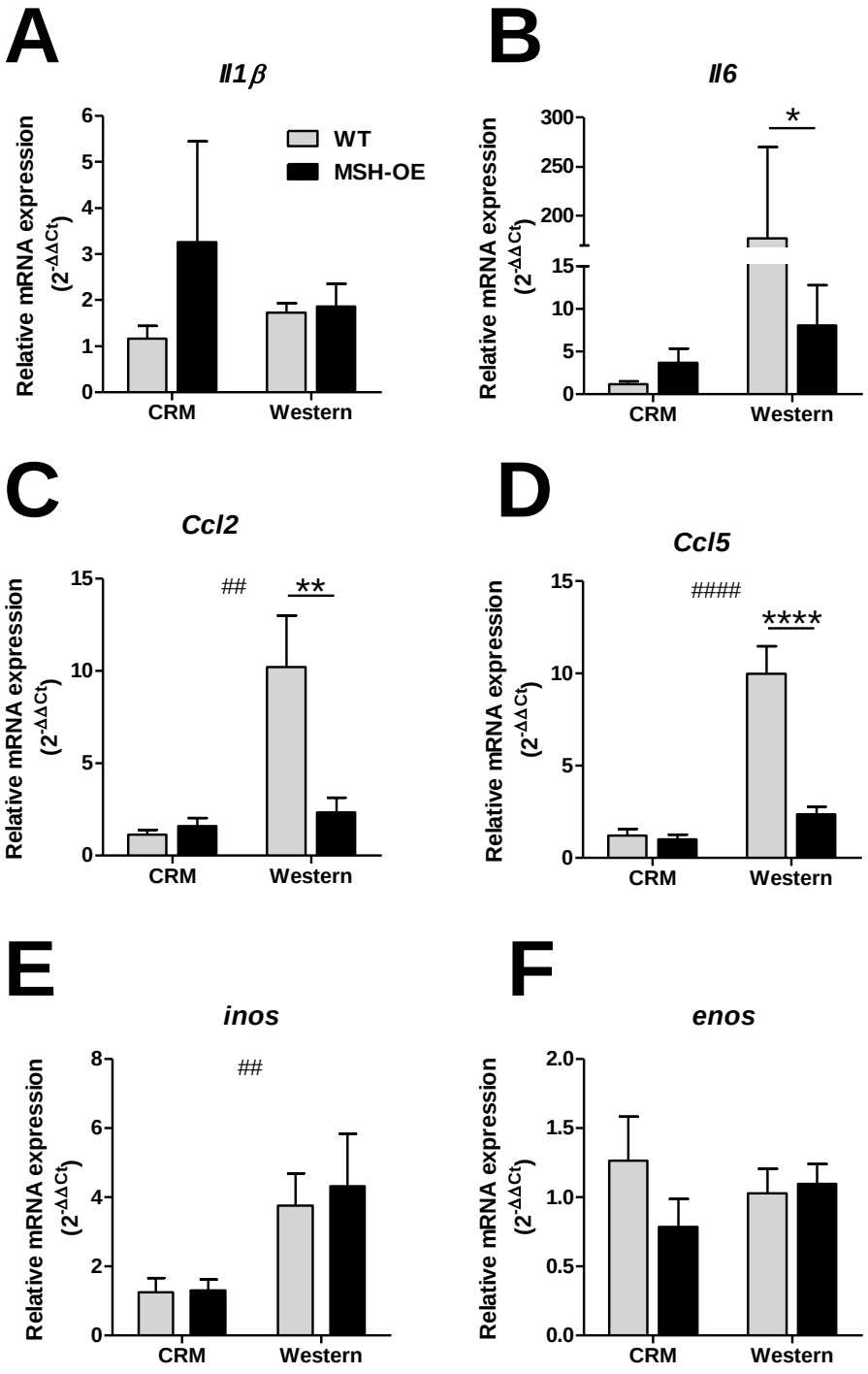

Fig. 19. Relative mRNA expressions of inflammatory markers in the thoracic aorta of WT and MSH-OE mice on regular CRM and Western diet. Western diet increased the expressions of *Ccl*2, *Ccl5* and *inos* (## P<0.001, #### P<0.00001 for the diet effect). On Western diet, α- and γ_3-MSH-OE decreased the expressions of *Il6*, *Ccl2* and *Ccl5* (* P<0.05, ** P<0.001, **** P<0.00001 for the genotype effect). Statistical differences were calculated using 2-way ANOVA followed by Bonferroni *post hoc* tests. Values are mean±SEM, n=6 per group.

6 DISCUSSION

6.1 Methodological considerations

6.1.1 Animal model

The present study was conducted to determine the effects of the trangenic melanocortin OE in atherosclerosis. Transgenic mice overexpressing α- and γ_3-MSH and deficient in Ldlr were used as an experimental model. Ldlr deficiency in mice is analogous to human familial hypercholesterolemia that is caused by either heterozygous or homozygous LDLR deficiency. The prevalence of the homozygous form is very rare, affecting only about 1 in 160 000 to one million people worldwide, whereas heterozygous mutation in LDLR is 1 in 200 or 500 worldwide, so it is relatively common (The FH Foundation 2015). Although the homozygous *Ldlr*$^{-/-}$ model is not perfectly comparable to human situation, it provides a useful tool for studying atherosclerosis in mice because normally they have low cholesterol levels and a protective lipoprotein profile, and therefore, do not normally develop atherosclerosis (Mills, Taylaur 1971). Furthermore, atherogenic diet consisting of high fat and cholesterol promotes the disease development so that *Ldlr*$^{-/-}$ mice develop major lesions already after 4 months on the diet. This, of course, accelerates the research when life-long animal studies are impractical to conduct. However, the situation is relatively different in humans where the development of atherosclerosis can take decades. In addition, *Ldlr*$^{-/-}$ mice rarely demonstrate plaque ruptures, which are the most serious clinical events in humans.

Despite its limitations, this transgenic mouse provides major advantages over conventional pharmacological models, including stable MSH levels without frequent and tightly scheduled peptide injections. Because there is no need to inject the peptides and adjust the dose for each animal, the possibility of human mistakes, which again might alter the peptide levels, is lower. Moreover, the injections cause additional stress to the animals. In addition, transgenic approach provides a life-long α- and γ_3-MSH-OE "administration" that would not be feasible to conduct using conventional MSH peptide administration. However, because the transgene comprises both α- and γ_3-MSH, a limitation of this model is that it is not possible to distinguish the contributions of α- and

γ_3-MSH and the respective receptors to the observed anti-inflammatory and vasoactive effects. Given that both α- and γ_3-MSH display these effects, it is likely that they both contribute to the observed phenotype. The receptors for α- and γ_3-MSH, namely MC1R and MC3R, are widely distributed in the periphery and in the CNS, and as such, the therapeutic effects of α- and γ_3-MSH-OE are likely to be mediated via both peripheral and central mechanisms. Recently, it was shown that deficiency of either MC1R or MC3R disturbs the anti-inflammatory signalling (Holloway, Durrenberger et al. 2015, Rinne, Ahola-Olli et al. 2015). However, it seems that MC3R plays a more significant role in the acute inflammatory response, whereas MC1R contributes more to the delayed immune response (Holloway, Durrenberger et al. 2015). Because both MC1R and MC3R have shown advantageous effects in inflammation and cardiovascular regulation, from a drug development point of view, it might be more beneficial to develop dual-agonists that would have more diverse therapeutical effects.

6.1.2 Wire-myography

The method of studying contractile and mechanical properties of the arteries, wire-myograph, was first described in 1977 by Mulvany and Halpern and has since been used to study various ring preparations (Mulvany, Halpern 1977). In the current study, wire-myograph was used to evaluate the functional and mechanical changes in the atherosclerotic aorta. The reason of studying aortae instead of any other vessel segment was that atherosclerosis is a disease that manifests in the large and medium-sized arteries. In addition, the aorta is the largest artery is mice, which simplifies the mounting procedure.

The standard procedure of preparing the arteries for the myograph apparatus aims at keeping the vessels functional for the experiments. Although the mounting procedure is performed carefully, it causes stress upon the aortae, which can affect their function.

Wire-myography is an *ex vivo* analysis method, and thus, has a great analytical power (Angus, Wright 2000). In a wire-myograph system, ring preparations can be studied for over 12 hours, which is impossible *in vivo*. In the absence of circulating hormones and neuronal innervations, every aortic ring can be studied without the interference of homeostatic mechanisms (Angus, Wright 2000). This enables the evaluation of the

underlying pathological mechanisms more accurately than *in vivo*. However, without the surrounding tissue and circulating hormones, the results obtained from the isolated arteries should be carefully considered before extrapolating them to *in vivo* situation.

6.2 Metabolic changes in MSH-OE mice

In this thesis, we carefully monitored the body weight development and body composition during the study. In males, α- and γ_3-MSH-OE had no effect on body weight development during the diet-intervention, whereas in females, α- and γ_3-MSH-OE tended to limit the Western diet induced body weight gain. This tendency in weight loss might result from decreased gonadal WAT mass that was almost statistically significantly decreased in MSH-OE mice compared with WT. α-MSH is an anorexic peptide, so it decreases the appetite and increases the energy expenditure. Similarly, [D-Trp^8]-γ-MSH has been shown to decrease food intake, although versatile results exist (Marks, Hruby et al. 2006, Lee, Kim et al. 2008). Because there were no differences in body weights between MSH-OE and WT mice, it can be concluded that the beneficial vasoactive and anti-inflammatory effects were not due to the weight loss effect of α- and γ_3-MSH. Considering that obesity is a major risk factor for cardiovascular diseases, weight-reducing therapy would be beneficial especially for those patients at risk for cardiovascular event.

Furthermore, α- and γ_3-MSH-OE decreased the relative liver weight in male mice on Western diet, opposite to female mice, whose relative liver weights were increased. These results most probably stem from accumulated fat deposits in the liver caused by Western diet. The accumulation of fat deposits in the liver impairs the normal function of the liver, including fat and glucose metabolism. This state called steatohepatitis is a major risk factor for atherosclerosis. In MSH-OE males, it could be speculated if decreased relative liver weight was due to decreased fat accumulation caused by α- and γ_3-MSH-OE. However, the absolute liver weights were unchanged. The differences in relative liver weights between males and females might stem from differencent suscetibilities in the hepatic lipid accumulations.

6.3 Vascular properties in MSH-OE mice

One of the objectives of this thesis was to study whether transgenic α- and γ_3-MSH-OE ameliorates vascular function and mechanical properties of the aortae in atherosclerosis. During the development of atherosclerosis, endothelial damage and dysfunction are observed in the vasculature even before the plaque formation. The damaged endothelium changes the tone of the vasculature so that vasoconstriction predominates. (Davignon, Ganz 2004) This pathological change was also observed in this project. α- and γ_3-MSH-OE had no effect on the potassium-evoked vasoconstriction, but it resisted the phenylephrine-induced contractions, and thus, improved the function of the aorta. α- and γ_3-MSH-OE also enhanced the endothelium-dependent vasorelaxation when mice were fed regular CRM diet. Most probably these effects were absent in Western group because the diet causes major endothelial dysfunction that overrules the beneficial effects of α- and γ_3-MSH-OE. Because eNOS is mainly responsible for the NO synthesis in the vascular endothelium, we hypothesized that *enos* would be upregulated in the aortae of MSH-OE mice. Since its expression was unchanged, the enhanced endothelium-dependent vasodilation most likely originated from other mechanisms than increased transcription. Previously, Rinne et al. showed that α-MSH stimulates phosphorylation of eNOS at Ser1177 residue and decreases oxidative stress-mediated NO breakdown, which together augment NO availability (Rinne, Nordlund et al. 2013, Rinne, Ahola-Olli et al. 2015). The present and previous evidence demonstrate that α- and γ_3-MSH-OE ameliorates endothelial dysfunction by promoting endothelium-dependent vasodilation without directly modifying *enos* expression. In the present study, α- and γ_3-MSH-OE had no effect on the aortic distensibility, although we had showed previously that *Mc1r*-deficiency stiffens the aorta (Rinne, Ahola-Olli et al. 2015).

6.4 Plaque characteristics in MSH-OE mice

This thesis showed that α- and γ_3-MSH-OE significantly decreased the plaque accumulation in the aortic arch. On the contrary to our previous study, where we treated the atherosclerotic mice for 4 weeks with MT-II, there was no difference in the plaque size (Rinne, Silvola et al. 2014). However, this difference in results most probably stems from the animal models used, i.e. melanocortin administration *versus* transgenic MSH-OE. Although MT-II is a very potent α-MSH analogue and the treatment duration of 4 weeks was able to constrain the development of atherosclerosis, this duration might

be insufficient to limit the plaque accumulation, when the mice had already developed atherosclerosis before the treatment initiation. In the present study, on the other hand, the transgenic α- and γ_3-MSH-OE provided a life-long MSH "treatment", and therefore, might be more efficient in limiting and even preventing the plaque accumulation at the early stage of the disease development. Moreover, the transgenic model provides consistent and stable activation of the melanocortin system, whereas the administration of MSH peptides requires frequent i.p. injections and therefore the level of MSH peptides might vary significantly throughout the day, which again might decrease the therapeutic effects.

Apart from plaque size, the clinical outcome in atherosclerosis is determined by the composition of the plaque. As the disease progresses, MMPs degrade the fibrotic tissue as an adaptive response to decreased lumen area, which thins the collagen cap and makes it susceptible to rupture. As a marker for plaque instability, we determined the collagen deposition and found that α- and γ_3-MSH-OE did not decrease the collagen deposition, i.e. α- and γ_3-MSH-OE had no effect on plaque instability. Given that α-MSH and *Mc1r*-deficiency have been shown to modulate collagen synthesis (Böhm, Stegemann 2014, Lorenz, Seebach et al. 2014, Rinne, Ahola-Olli et al. 2015), the differences in our results might yield from insufficient sample quality. Moreover, the plaque accumulation decreases when receding from the aortic root, and therefore, the differences in section points in relation to aortic valves might distort the results. In this project, Masson's Trichrome staining were performed only for Western group, which is why it is possible that pathological mechanisms in atherosclerosis override the beneficial effects of α- and γ_3-MSH-OE as mentioned above.

6.5 Anti-inflammatory properties in MSH-OE mice

The decreased plaque accumulation in MSH-OE mice is well in line with the reduced cytokine expression levels observed in this study. *Ccl2* deficiency or polymorphism decreases the plaque burden both in mice and humans (Wan, Murphy 2013). The atherogenic effects of CCL2 stem from its ability to recruit monocytes into the inflammatory site; the effect that is abolished in Ccr2-deficient mice (Boring, Gosling et al. 1997, Boring, Gosling et al. 1998). Similarly, Ccr5-deficient bone transplantation in mice decreases the plaque burden and monocyte trafficking to the site of inflammation

(Potteaux, Combadiere et al. 2006, Braunersreuther, Zernecke et al. 2007). In addition to *Ccl2* and *Ccl5*, *Il6* was also decreased in the aorta of MSH-OE mouse. In another atherosclerotic mouse model, in *Apoe*$^{-/-}$ mouse, *Il6* expression is also significantly increased (Sukovich, Kauser et al. 1998); the effect that we also observed in our *Ldlr*$^{-/-}$ mouse. IL6 is secreted by macrophages in the atherosclerotic plaques; and especially, macrophages loaded with free cholesterol are a major source of IL6 (Sukovich, Kauser et al. 1998). Although IL6 has been considered as an atherogenic molecule, some studies indicate that IL6 would be atheroprotective (Van Lenten, Wagner et al. 2001, Schieffer, Selle et al. 2004).

Our findings show that α- and γ_3-MSH-OE was able to decrease the proportion of a M1 macrophage marker iNOS in the aortic root. M1 macrophages feed plaque inflammation and promote vulnerability by secreting pro-inflammatory markers, such as iNOS. In the present study we show that α- and γ_3-MSH-OE decreased the iNOS-positive area in the aortic root. This might be an indication of the ability of α- and γ_3-MSH-OE to shift the macrophage phenotype towards anti-inflammatory M2 type (Rinne, Silvola et al. 2014). Still, more research is needed to confirm the switch of macrophage phenotype by α- and γ_3-MSH-OE. M1 macrophages secrete proinflammatory cytokines, including IL1 and IL6. Supporting the iNOS findings, *Il6* expression was significantly suppressed in MSH-OE mice, possibly due to reduced M1 macrophage deposition. Moreover, other pro-inflammatory markers *Ccl2* and *Ccl5* that recruit leukocytes were downregulated in MSH-OE mice (Wan, Murphy 2013). Recently, Yang et al. showed that α-MSH was able to reduce the infiltration of monocytes by downregulating the expression of VCAM-1 that also promotes the arrest of monocytes and T cells in the endothelium (Cybulsky, Iiyama et al. 2001, Yang, Zhang et al. 2015). Given that the endothelium and endothelium-derived NO play a crucial role in controlling the leukocyte adhesion and cytokine secretion, it might be that the anti-inflammatory actions of melanocortins are governed by enhanced NO bioavailability (Rinne, Silvola et al. 2014). Morover, NO protects the vasculature by restraining smooth muscle cell proliferation, LDL oxidation and inflammatory responses, which are all compromised in atherosclerosis (Davignon, Ganz 2004).

6.6 Future perspectives

In this thesis, the long-term activation of the melanocortin system in a transgenic mouse model showed therapeutical potential in protecting against atherosclerosis. As an endogeneous peptide system, the melanocortin system regulates a wide range of homeostatic functions, including immune and cardiovascular system. Due to these characteristics, the melanocortin system appears as an ideal drug development target for atherosclerosis, an inflammatory disease of the arteries. However, owing to such wide range of functions, activation or deactivation of this system is unlimited to certain tissues or cells. Furthermore, MCRs are widely distributed in the body, including the CNS, and hence targeting these receptors might yield in unwanted effects on, for example, sexual functions or energy homeostasis. The discovery and development of melanocortin ligands that would be specific to the tissue- or receptor-of-interest might reduce the side effects and induce the beneficial effects. Still, these results should be carefully examined before further extrapolation to humans and more research should be conducted to evaluate the definitive therapeutic potential of the melanocortinergic activation in atherosclerosis treatment and prevention.

7 CONCLUSIONS

This study using a transgenic MSH-OE mouse model demonstrates that α- and γ_3-MSH-OE is able to alleviate the progression of atherosclerosis in mice. The main conclusions of this thesis are:

1. Trangenic α- and γ_3-MSH-OE limits the plaque accumulation in the aortic arch when the mice are challenged with Western-style diet.
2. Transgenic α- and γ_3-MSH-OE alleviates the endothelial dysfunction in atherosclerosis by shifting the vascular tone towards vasodilatation instead of vasoconstriction that is often observed in atherosclerosis.
3. Trangenic α- and γ_3-MSH-OE limits the diet-induced elevation of the pro-inflammatory cytokines that contribute to the pathogenesis of atherosclerosis.

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