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Methotrexate Enhances Atherosclerosis Progression *via* Impairment of Folate Pathway in a Microminipig Model

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Abstract

Background/Aim: As the pathophysiology of Microminipigs (μMPs) is similar to that of human, μMPs are useful in atherosclerosis research. To clarify the effect of methotrexate (MTX) on atherosclerosis, we investigated the pathology of MTX-induced atherosclerosis lesion exacerbation in μMPs fed a high-fat and high-cholesterol diet (HFHCD).

Materials and Methods: The μMPs were divided into four groups: HFHCD, HFHCD+MTX, HFHCD+MTX+leucovorin (LV), and HFHCD+MTX+folic acid (FA), and fed for two weeks. Laboratory tests including blood lipid, FA, and homocysteine (Hcy) levels, and pathological evaluation of the atherosclerosis lesion area and thickness were performed. Hepatic and jejunal gene expressions related to lipid and folate metabolism pathways including 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR) were monitored using RT-PCR.

Results: The HFHCD+MTX group showed increased blood Hcy ($p<0.01$) and decreased FA levels ($p<0.05$) in accordance with increased hepatic MTR mRNA expression ($p<0.1$) and exacerbation of atherosclerosis ($p=0.051$ for lesion area and $p=0.045$ for lesion thickness) compared to the HFHCD group. Administration of LV or FA attenuated the MTX-induced increase in the Hcy level ($p<0.01$), atherosclerosis lesion thickness ($p<0.1$), and MTR mRNA expression ($p<0.1$ in HFHCD+MTX *vs*. HFHCD+MTX+LV groups).

Conclusion: MTX exacerbated HFHCD-induced atherosclerosis mediated through reduced blood FA and the subsequent increase of Hcy in μMPs, indicating that the μMP model may advance cardio-oncology research by providing useful experimental approaches. As MTX is administered for rheumatoid arthritis and malignant tumors in humans, atherosclerosis exacerbation should be acknowledged as a possible adverse effect of MTX treatment.

Keywords: Atherosclerosis, animal model, microminipig, high-fat and high-cholesterol diet, methotrexate, hyperhomocysteinemia, folic acid, leucovorin.

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Introduction

Atherosclerosis is a predominant risk factor in cardiovascular and cerebrovascular events and closely related to serious morbidity (1). Myocardial and cerebral infarction due to arterial atherosclerosis are major causes of death, and result in a discrepancy between average and healthy life expectancy (2). Folic acid (FA) deficiency causes blood homocysteine (Hcy) elevation, a risk factor for atherosclerotic vascular diseases in humans (3, 4). Therefore, blood Hcy levels can be used as prognostic indicators along with non-high-density lipoprotein (HDL)-cholesterol levels (5, 6). Furthermore, blood Hcy levels are high in countries with low folic acid intake (7). Epidemiological studies also demonstrated that increased blood Hcy (hyperhomocysteinemia) is a risk factor for human infarct diseases (8-11). Methotrexate (MTX), a widely used drug for cancer and rheumatoid arthritis (RA) patients, is an FA metabolism antagonist that reduces blood FA levels (12, 13), suggesting that MTX could exacerbate atherosclerosis. However, conversely, MTX reportedly protects against atherosclerosis and cardiovascular events in RA patients (14, 15). In addition to clinical cohort studies, experimental approaches using appropriate animal atherosclerosis models are required to resolve whether MTX is actually atheroprotective or proatherogenic.

To investigate the pathogenesis of atherosclerosis, appropriate animal models that reproduce human physiology and pathology are required. Mice and rabbits were widely used to develop animal models of atherosclerosis, but lipid metabolism in mice is high-density lipoprotein (HDL)-cholesterol dominant and typically resistant to a high-fat/high-cholesterol diet (HFHCD). Therefore, gene knockout is necessary to cause atherosclerosis in mice (16, 17). Rabbits are more sensitive to HFHCD for the introduction of atherosclerosis, and have a similar lipid metabolism to humans. The use of low-density lipoprotein receptor (*LDLr*) gene-mutated Watanabe heritable hypercholesterolemia and transgenic rabbits clarified the effects of specific genes on the development of atherosclerosis (18-20). However, swine provide useful animal models, because their physiology and style of feeding and sleep are similar to those of humans, leading to the development of many swine strains for experimental uses, including atherosclerosis research (21). Among them, the Microminipig™ (μMP, Fuji Micra, Shizuoka, Japan) has been established as the world's smallest experimental minipig (22). Our previous studies demonstrated that μMPs are very sensitive to HFHCD and prone to develop hypercholesterolemia-induced atherosclerosis (23-25).

In the present study, we investigated the potential for MTX to exacerbate atherosclerosis in a 2-week μMP hyperlipidemia-induced atherosclerosis model. As FA are administered to prevent FA antagonistic toxicity of MTX in RA patients (26), leucovorin (LV) and FA are used to realize a reduction of MTX-induced blood Hcy elevation and atherosclerotic lesions. Herein, we demonstrated that MTX accelerated atherosclerosis *via* decreased FA and a subsequent increase of blood Hcy in μMP models. These results indicate that short-term MTX administration would have the adverse effect of atherosclerosis lesion exacerbation. The present study using the μMP atherosclerosis model would promote cardio-oncology research by providing an essential tool for studying atherosclerosis associated with anticancer treatment.

Materials and Methods

Animal maintenance. Male μMPs were obtained from a breeder (Fuji Micra) and maintained in animal rooms at a temperature of 20-26˚C and relative humidity of 30-70%, with a 12-h light/dark cycle. Tap water was available *ad libitum*. All experimental protocols were approved by the Ethics Committee of Animal Care and Experimentation, Kitasato University (21-016) and HAMRI Co., Ltd. (IB20032). The research was performed in accordance with the Institutional Guidelines for Animal Experiments and in compliance with the Japanese Law Concerning the Protection and Control of Animals (Law No. 105 and Notification No. 6).

Table I. *Body weight and visceral and subcutaneous adiposity in the μMP model.*

Items		Treatment group			
		HFHCD	HFHCD+MTX	HFHCD+MTX+LV	HFHCD+MTX+FA
Dose (mg/body/day)	MTX (p.o.)	–	3.3	3.3	3.3
	LV (i.m.)	–	–	0.99	
	FA (i.m.)	–	–	–	4.5
Body weight (kg)[a]					
Day 1		10.9±0.6	10.9±0.4	10.0±04[#]	10.3±0.6
Day 14		12.1±1.1	12.5±0.7	11.0±0.8	11.7±0.7
Body weight gain (kg)[a]		1.1±0.5	1.7±0.3	1.0±0.5	1.4±0.2
Body weight gain (%)[a]		109.8±4.7	115.1±2.4	109.7±4.3	113.1±1.8
Relative adiposity weight (g/kg)[a]					
Greater omentum		0.9±0.2	1.3±0.3	0.6±0.2[#]	1.2±0.4
Mesenterium		2.3±0.6	2.6±0.8	2.2±0.2	3.2±1.0

–: Non-treatment, [a]Mean±standard error. [#]$p<0.1$ *vs.* HFHCD+MTX. HFHCD: High-fat and high-cholesterol diet; MTX: methotrexate; LV: leucovorin; FA: folic acid; μMP: Microminipig.

Study design. Twelve μMPs (6-7 months old, 10.6±0.85 kg BW) were divided equally into four groups and each fed HFHCD for 2 weeks, involving 300 g diet a day ingested in the morning. The HFHCD diet was composed of fat (3.6 g/kg/day, refined lard; Miyoshi Oil & Fat Co., Tokyo, Japan) and cholesterol (0.15 g/kg/day, Wako Pure Chemical Industries, Osaka, Japan) mixed with a regular diet (Marubeni Nisshin Feed, Tokyo, Japan), as previously reported (23). In HFHCD+MTX, HFHCD+MTX+LV, and HFHCD+MTX+FA groups, MTX (3.3 mg/body/day), LV (0.99 mg/body/day), and FA (4.5 mg/body/day) were administered intramuscularly in the morning, respectively (Table I). LV, also known as folic acid, is an active form of folic acid that does not require activation by dihydrofolate reductase.

Laboratory tests. Clinical observation was conducted every day, and body weight was measured on the first and 14th days of the experiment. Peripheral blood samples were collected from the cranial vena cava at Days 1 and 14 for hematology and biochemistry. Biochemical parameters included aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, lactate dehydrogenase, γ-glutamyl transpeptidase, total bilirubin, and glucose. Levels of total cholesterol (TC), very low-density lipoprotein-cholesterol (VLDL-C), low-density lipoprotein-cholesterol cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C), and triglycerides (TG) were analyzed using an automated apparatus (LABOSPECT 008, Hitachi High-Tech Corporation, Tokyo, Japan; DM-JACK, Minaris Medical Co., Ltd., Tokyo, Japan). After the 2-week experiment, all μMPs were anesthetized, sacrificed by bilateral axillary artery exsanguination, and necropsied. The weight of visceral fat (greater omentum and mesenterium) was measured and calculated as the relative adiposity weight [fat weight (g)/BW (kg)].

Pathological examination. At necropsy, the aorta, heart, liver, kidneys, spleen, and intestine were resected for pathological examination. The heart, liver, kidneys, spleen, and visceral adipose tissue (greater omental and mesenteric adipose tissues) were weighed. All organs were fixed in 10% phosphate-buffered formalin and embedded in paraffin for routine histological examination. The thoracic and abdominal aortas were cut open longitudinally and stained with Oil Red O stain. En face images of the aortas were captured using a digital camera, and the Oil Red O-positive area relative to the whole surface area was calculated using Image J software (23, 24). Formalin-fixed, paraffin-embedded aortas were processed for hematoxylin and eosin (H&E) and Elastica Masson stains. The abdominal aorta was

Table II. *Genes investigated and primers used for PCR.*

Gene	Assay ID	Ref. Seq.	GenBank
LDLr	Ss03374441_u1	NM_001206354.2	AF065990.1
HMGCR	Ss03390147_m1	NM_001122988.1	DQ432054.1
SREBP-2	Ss03376492_u1	XM_021091444.1	AY493571.1
NPC1L1	Ss06874101_m1	XM_005673340.3	
APOBEC-1	Ss06858597_m1	XM_003126519.4	
MTR	Ss03373360_m1	XM_001927058.4	AF276463.1
CHDH	Ss06909601_s1	XM_005669647.3	AK393413.1
PEMT	Ss03384368_u1	NM_214365.1	AY334573.1
GAPDH	Ss03375629_u1	NM_001206359.1	AF439784.1

traversed at 3-mm intervals, and 17-24 sections/animal were subjected to pathological examination. Then, three of the most severe lesion sites in the abdominal aorta were arbitrarily selected, and the thickness of each lesion site was measured at three locations, wherein the lesion thickness was averaged to give a value of one individual. Immunostaining for abdominal atherosclerotic lesions was carried out for paraffin sections using antibodies against smooth muscle actin (anti-α-SMA clone 1A4, ×50; Dako, Tokyo, Japan) and macrophages (anti-lysozyme rabbit polyclonal antibody, ×25; Dako, Tokyo, Japan).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR). The liver and jejunum were collected immediately following necropsy in all animals (n=3/group). Small pieces of fresh tissues (~3 mm^3) were placed in RNAlater (Sigma-Aldrich, Tokyo, Japan) at 4˚C overnight, and then stored at –30˚C until use. Total RNA was extracted from the liver and jejunum using RNeasy Mini Kit (Qiagen, Hilden, Germany), and mRNA expression of *LDLr*, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*HMGCR*), sterol regulatory element binding protein 2 (SREBP2), Niemann-Pick C1-Like 1 (*NPC1L1*), 5-methyltetrahydrofolate-homosystein methyltransferase (*MTR*), choline dehydrogenase (*CHDH*), and phosphatidylethanolamine N-methyltransferase (*PEMT*) in the liver was quantified using qRT-PCR. Expression of jejunal apolipoprotein B-100 RNA editing catalytic subunit-1 (*APOBEC-1*) mRNA was also quantified using qRT-PCR (23, 24). The expression level of glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. Primers used for RT-PCR are listed in Table II.

Statistics. All results are expressed as the mean±standard error. Differences were analyzed using *t*-tests. Differences were considered significant at $p<0.05$ and borderline-significant at $p>0.05$ and <0.1.

Results

General observation. No abnormal manifestations were observed in any of the groups during the experimental period. The final BW gains (from Day 1 to Day 14, with starting experimental value of 100%) in all groups were approximately 10% (Table I). BW gains and relative adiposity weights showed no significant differences among the four groups, except for between HFHCD+MTX and HFHCD+MTX+LV groups, with $p=0.092$ and $p=0.065$, respectively (Table I). There were no significant differences in mean absolute or relative organ weights.

Laboratory tests. Blood levels of TC, LDL-C, HDL-C, and free-C increased rapidly during the 2 weeks in all groups. TC, LDL-C, and free-C levels increased markedly in the HFHCD group than in the other groups; however, no significant statistical differences were observed (Figure 1). Blood levels of hepatic enzymes showed no significant differences among the four groups, and were within the reference data

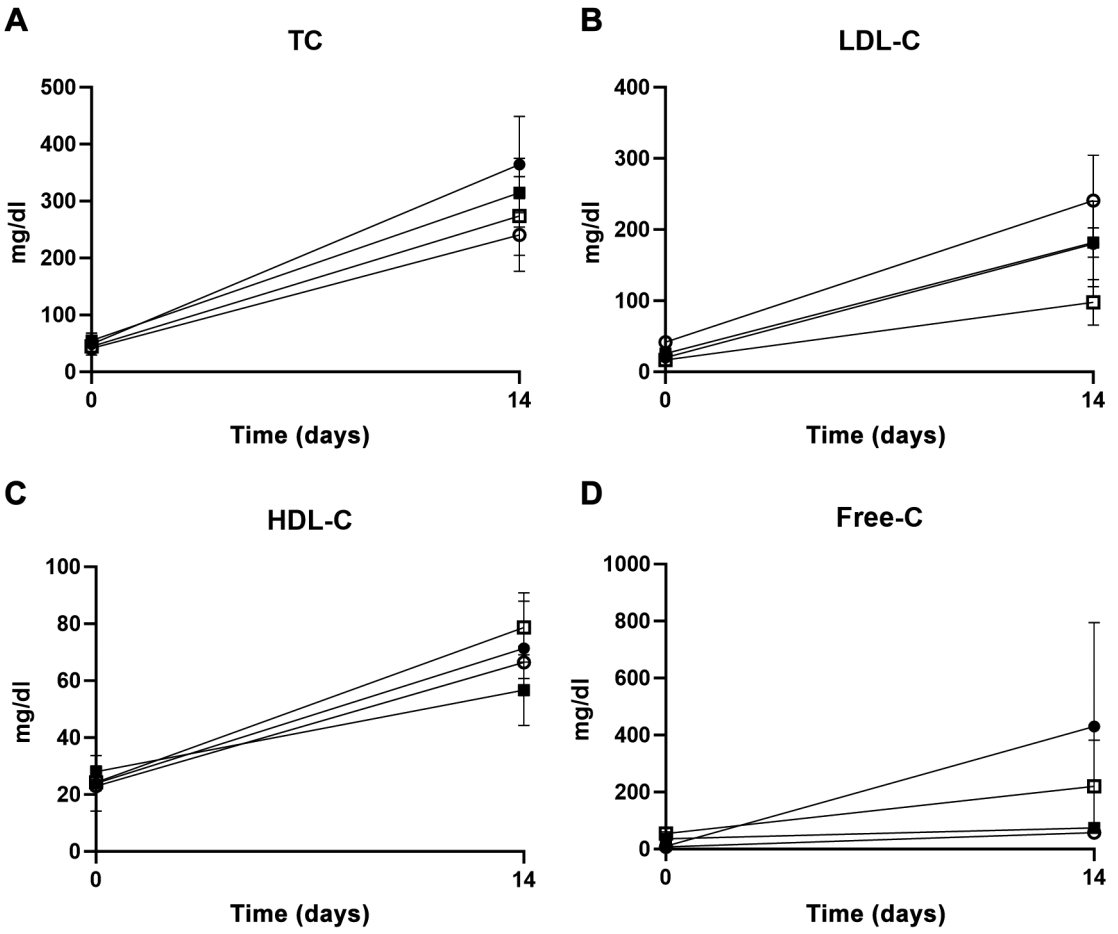

Figure 1. *Serum levels of lipid metabolism markers. (A) TC, (B) LDL-C, (C) HDL-C, (D) Free-C. Blood levels of all markers increased rapidly during the 2 weeks in all groups. Open circle: HFHCD, closed circle: HFHCD+MTX, open rectangle: HFHCD+MTX+LV, closed rectangle: HFHCD+MTX+FA. TC: Total cholesterol; LDL-C: low-density lipoprotein-cholesterol, HDL-C: high-density lipoprotein-cholesterol, Free-C: free cholesterol.*

on μMPs (27, 28). The blood FA concentration in the HFHCD+MTX group was lower than that in the HFHCD group (p=0.025), while the concentrations in HFHCD+MTX+LV and HFHCD+MTX+FA groups were comparable with those in the HFHCD group (Figure 2A). The blood Hcy level in the HFHCD+MTX group was significantly higher than in all other groups (p<0.01). Hcy levels in HFHCD+MTX+LV and HFHCD+MTX+FA groups were moderately decreased compared with the HFHCD group (p<0.05) (Figure 2B). These results indicate that additional administration of LV and FA attenuated the MTX-induced blood Hcy elevation.

Pathological examination. None of the animals showed grossly significant anomalies in any of the organs, except for the aortas and arteries. En face Oil red O staining of the thoracic and abdominal aortas stained linear and patchy atherosclerotic lesions red in color (Figure 3A). The lesion % area with positive Oil red O staining in the HFHCD+MTX group was larger than the HFHCD group (p=0.051, Figure 3B). The HFHCD+MTX group showed no significant differences in the lesion % area between HFHCD+MTX+LV and HFHCD+MTX+FA groups. In contrast, the thickness of atherosclerotic lesions in the aortas of the HFHCD+MTX group, which represent the most severe atherosclerotic

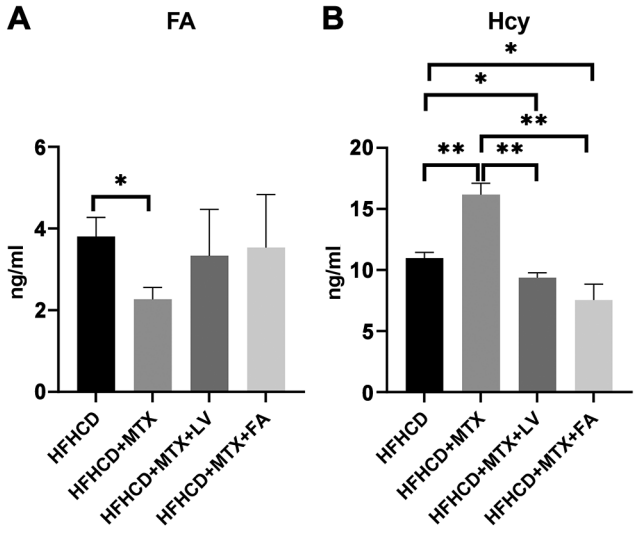

Figure 2. *Serum folic acid and homocysteine levels. (A) FA and (B) Hcy concentration at 2 weeks. In the HFHCD+MTX group, FA concentration decreased, and Hcy concentration increased compared to other groups. *p<0.05, **p<0.01. FA: Folic acid; Hcy: homocysteine; HFHCD: high-fat and high-cholesterol diet; MTX: methotrexate; LV: leucovorin.*

lesions, were thicker than in the HFHCD group (p=0.045) (Figure 3C). The lesion thickness in the HFHCD+MTX+LV and HFHCD+MTX+FA groups were thinner than in the HFHCD+MTX group, with p=0.074 and p=0.082, respectively (Figure 3C). The atherosclerotic lesions were localized in the arterial and aortic intima, which was thickened due to proliferation of lysozyme-positive macrophage-derived foam cells and α-SMA-positive smooth muscle cells (Figure 4A, B, and C). No lymphoproliferative diseases were observed in MTX-treated groups.

Study of gene expression. The expression level of hepatic *MTR* mRNA in the HFHCD+MTX group was higher than in the HFHCD group (p=0.085). The *MTR* mRNA expression level in the HFHCD+MTX+LV group was decreased in comparison with that in the HFHCD+MTX group (p=0.099), and similar to that in the HFHCD group. The *MTR* mRNA expression level in the HFHCD+MTX+FA group was comparable with that in the HFHCD group (Figure 5A). Expression levels of hepatic *CHDH* mRNA in the HFHCD group showed differences among HFHCD+MTX+LV (p=0.02), HFHCD+MTX (p=0.066), and HFHCD+MTX+FA groups (p=0.099) (Figure 5B). *PEMT* showed no significant differences among the four groups (Figure 5C). Expression of hepatic *LDLr* mRNA was high in the HFHCD group, but this was not statistically significant. A greater increase in *LDLr* mRNA expression was observed in the HFHCD+MTX+FA group compared to the HFHCD group (p=0.028) (Figure 6A). The expression of hepatic *NPC1L1* mRNA was higher in the HFHCD+ MTX+FA group than HFHCD group (p=0.098) (Figure 6B). Hepatic *HMGCR* and intestinal *APOBEC-1* mRNA expression levels showed no differences among the four groups (Figure 6C and D).

Discussion

Herein, we clearly reproduced our previous studies in which hypercholesterolemia-induced atherosclerosis developed in μMPs within 2 weeks of feeding them HFHCD (23-25). This is a much shorter experimental period to produce macroscopically identifiable atherosclerotic lesions than in other pig atherosclerosis models (29-32). To the best of our knowledge, this study is the first to demonstrate MTX-induced atherosclerosis exacerbation *via* impairment of folic acid metabolism in a hyperlipi-demic animal model.

HFHCD feeding increased TC, LDL-C, and free-C levels approximately 5-fold or more, as previously reported (23). MTX administration, however, led to no additional increase in blood lipid levels. However, MTX treatment decreased blood FA and increased Hcy with a subsequent increase of atherosclerosis lesions. Moreover, LV and FA treatments attenuated blood Hcy levels and atherosclerosis lesion thickness. These results indicate that MTX accelerates arteriosclerosis *via* increased blood Hcy (hyperhomocysteinemia). LV or FA supplementation as MTX antagonists administered to HFHCD+MTX-fed μMPs decreased atherosclerosis lesion thickness, but not lesion area, suggesting that LV or FA can suppress lesion progression of pre-existing atherosclerosis. As Hcy is a risk factor for worsening atherosclerosis in humans (8-11, 33), MTX medication is suggested to be a risk factor of atherosclerosis progression. The most important risk factor affecting atherosclerosis is blood LDL-C levels, which are mainly regulated by LDL-C

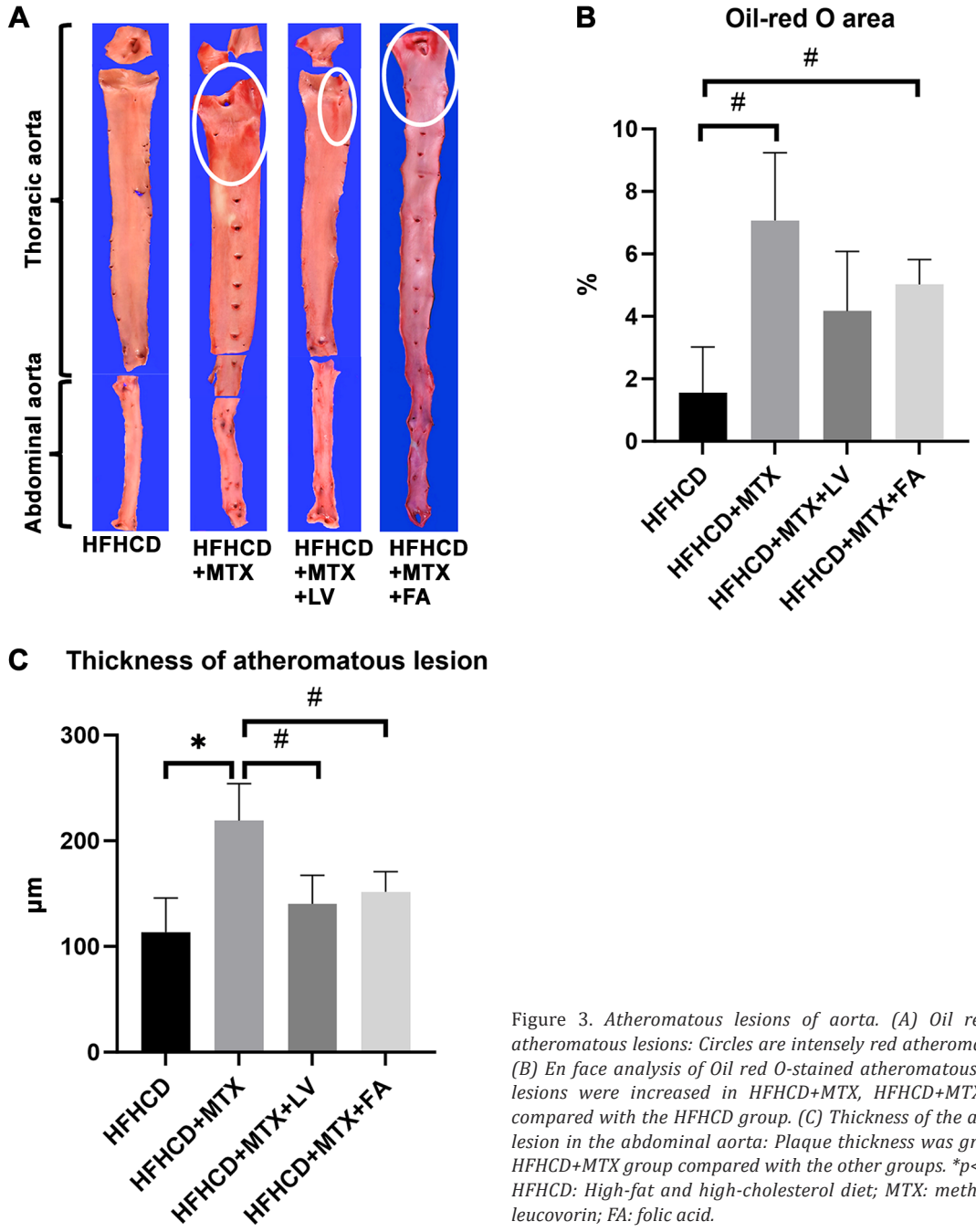

Figure 3. *Atheromatous lesions of aorta. (A) Oil red O-stained atheromatous lesions: Circles are intensely red atheromatous lesions. (B) En face analysis of Oil red O-stained atheromatous lesions: The lesions were increased in HFHCD+MTX, HFHCD+MTX+FA groups compared with the HFHCD group. (C) Thickness of the atheromatous lesion in the abdominal aorta: Plaque thickness was greatest in the HFHCD+MTX group compared with the other groups. *p<0.05, #p<0.1. HFHCD: High-fat and high-cholesterol diet; MTX: methotrexate; LV: leucovorin; FA: folic acid.*

removal *via* hepatic *LDLr* and cholesterol synthesis *via* hepatic *HMGCR* activity (34, 35). In the present μMP atherosclerosis model, MTX treatment did not change the *LDLr*, *HMGCR* mRNA, or blood lipid levels. MTX would exhibit no direct effects on lipid metabolism in μMPs; therefore, MTX-induced atherosclerosis progression would be mediated through hyperlipidemia-independent mechanism(s) in the present μMP model.

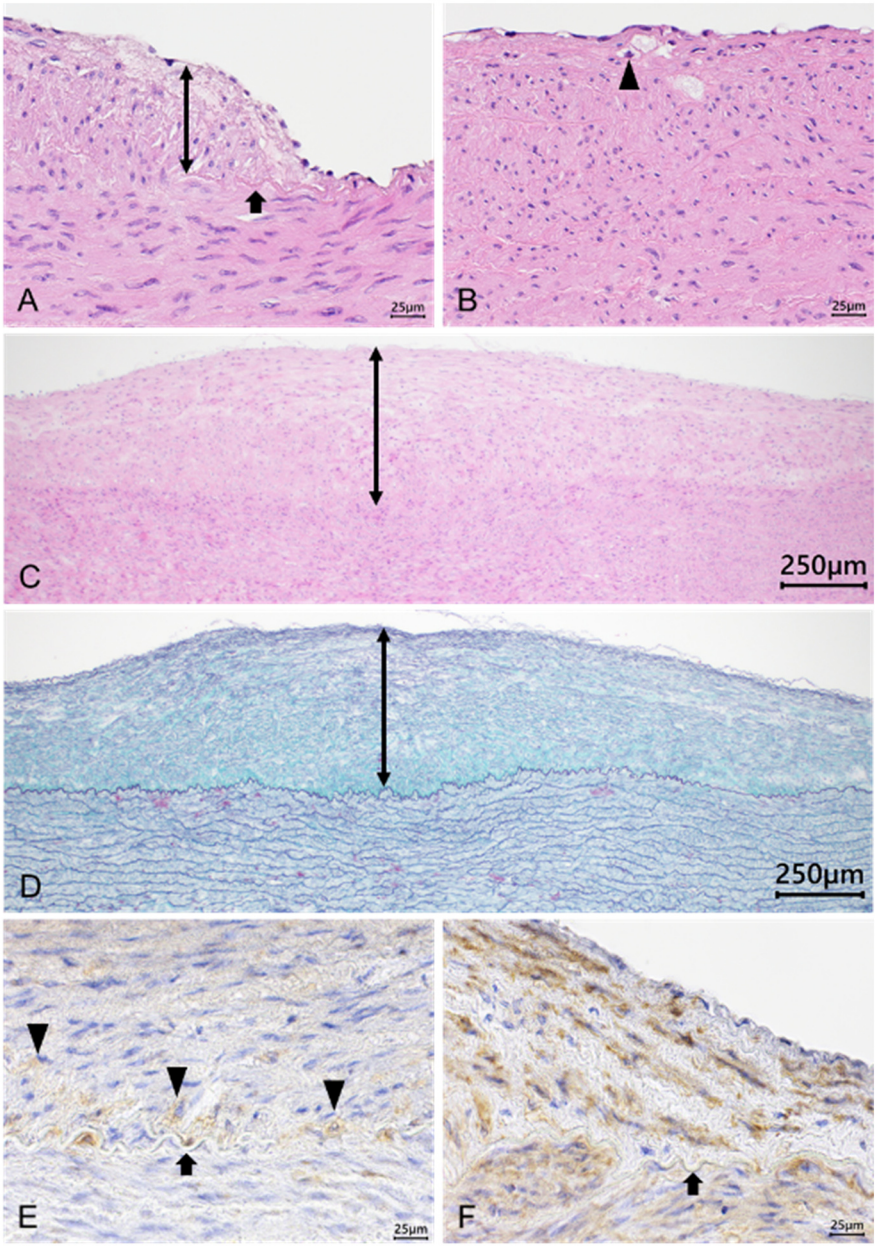

Figure 4. *Histopathological and immunohistochemical examinations of atherosclerotic lesions in HFHCD+MTX group. (A) Right coronary artery: Atheromatous plaque is shown. Arrow indicates internal elastic lamina. (B) Left coronary artery: Intimal infiltration of foam cells (arrowhead) is shown. (C, D) The abdominal aorta showed intimal fibrosis. (E) The lesion of intimal fibrosis included a few lysozyme-positive cells (arrowheads). (F) Some intima cells in the abdominal aorta were positive for α-SMA. A, B, and C: H&E stain, D: Elastica-Masson stain, E and F: Immunohistochemical stain. HFHCD: High-fat and high-cholesterol diet; MTX: methotrexate; H&E: hematoxylin and eosin.*

Some reports showed that treatment with FA and vitamin B12 is not associated with reduction of cardiovascular events (36, 37). In contrast, a meta-analysis demonstrated evidence for the beneficial effect of FA and vitamin B supplementations in reducing Hcy and preventing the combined risk of stroke, myocardial

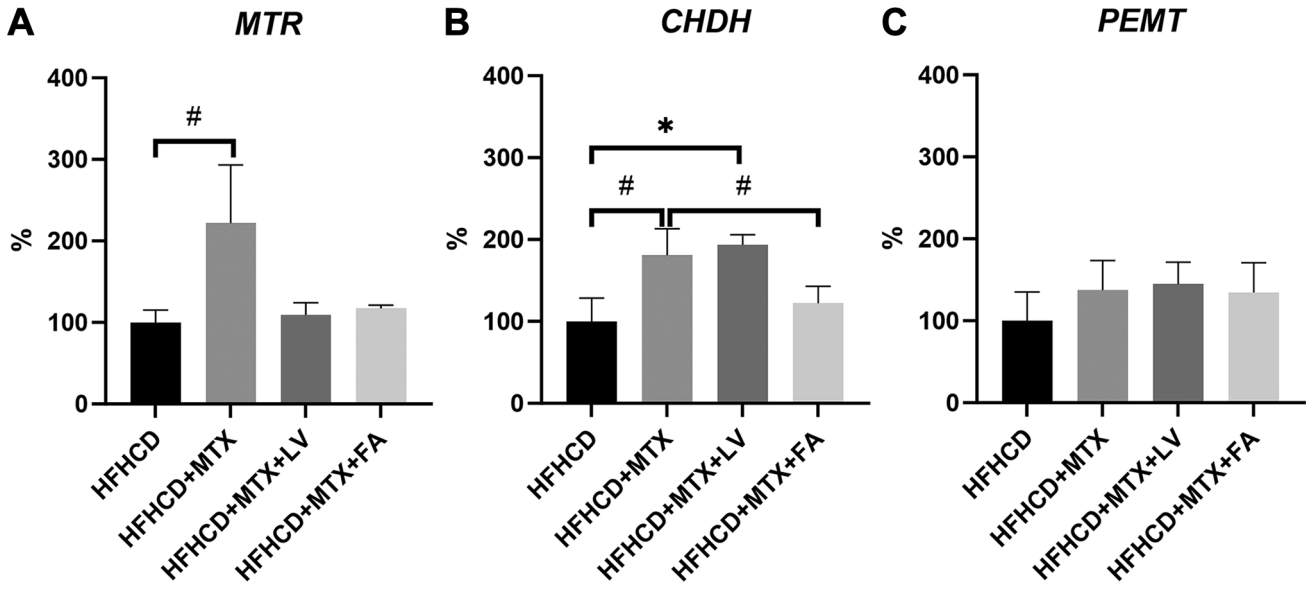

Figure 5. *Expression of hepatic MTR, CHDH, and PEMT mRNA. (A) MTR was up-regulated in the HFHCD+MTX group and down-regulated in HFHCD+MTX+LV and HFHCD+MTX+FA groups. (B) CHDH was up-regulated in HFHCD+MTX and HFHCD+MTX+LV groups and down-regulated in the HFHCD+MTX+FA group. (C) PMET: No difference was found among the 4 groups. *p<0.05, #p<0.1. MTR: 5-Methyltetrahydrofolate-homosystein methyltransferase; CHDH: choline dehydrogenase; PEMT: phosphatidylethanolamine N-methyltransferase; HFHCD: high-fat and high-cholesterol diet; MTX: methotrexate; LV: leucovorin; FA: folic acid.*

infarction, and vascular death (38). Although the preventive effects of FA on human atherosclerosis remain controversial, FA supplementation was found to reduce atherosclerotic lesion thickness in accordance with blood Hcy reduction in the μMP model. The expression of hepatic *MTR*, an enzyme that catalyzes the conversion of Hcy to methionine, was up-regulated in MTX-treated μMPs and down-regulated by LV and FA. This enzymatic activity requires 5-methyltetrahydrofolate (5-MTHF), a metabolite produced in the folate metabolic pathway. MTX primarily targets dihydrofolate reductase, reduces blood FA, and subsequently suppresses 5-MTHF production. Then, decreased 5-MTHF leads to increased Hcy *via* decreased *MTR* enzymatic activity. In the present study, *MTR* mRNA expression was affected by MTX, suggesting that the folate metabolic pathway would be comparable between humans and μMPs.

Clinically, MTX is a first-line treatment for RA patients (39). As the present study indicated, MTX increases blood Hcy levels *via* reduced blood FA (13, 40); therefore, MTX is suggested to be a risk factor for atherosclerosis and related cardiovascular events. For RA patients receiving MTX treatment, FA is administered as a supplement and this decreases Hcy levels and protects against cardiovascular events (15, 40). On the contrary, there are multimodal effects of MTX on anti-inflammation, decrease in proatherogenic lipoprotein(a), and improvement of endothelial functions that prevent atherosclerosis progression (41-44). Actually, MTX treatment is associated with a decreased cardio-vascular risk in RA patients (26, 44, 45). Conversely, the prescription of immunomodulatory drugs including MTX results in an increased risk of cardiovascular events of myocardial infarction, stroke, and cardiovascular death in patients with peripheral artery diseases (46). We cannot directly extrapolate the results obtained from our μMP model to human atherosclerosis pathology to conclude whether MTX is atheroprotective or proatherogenic. However, as one possible explanation, the discrepancy between clinical and experimental observations may be attributable to differences in the dose

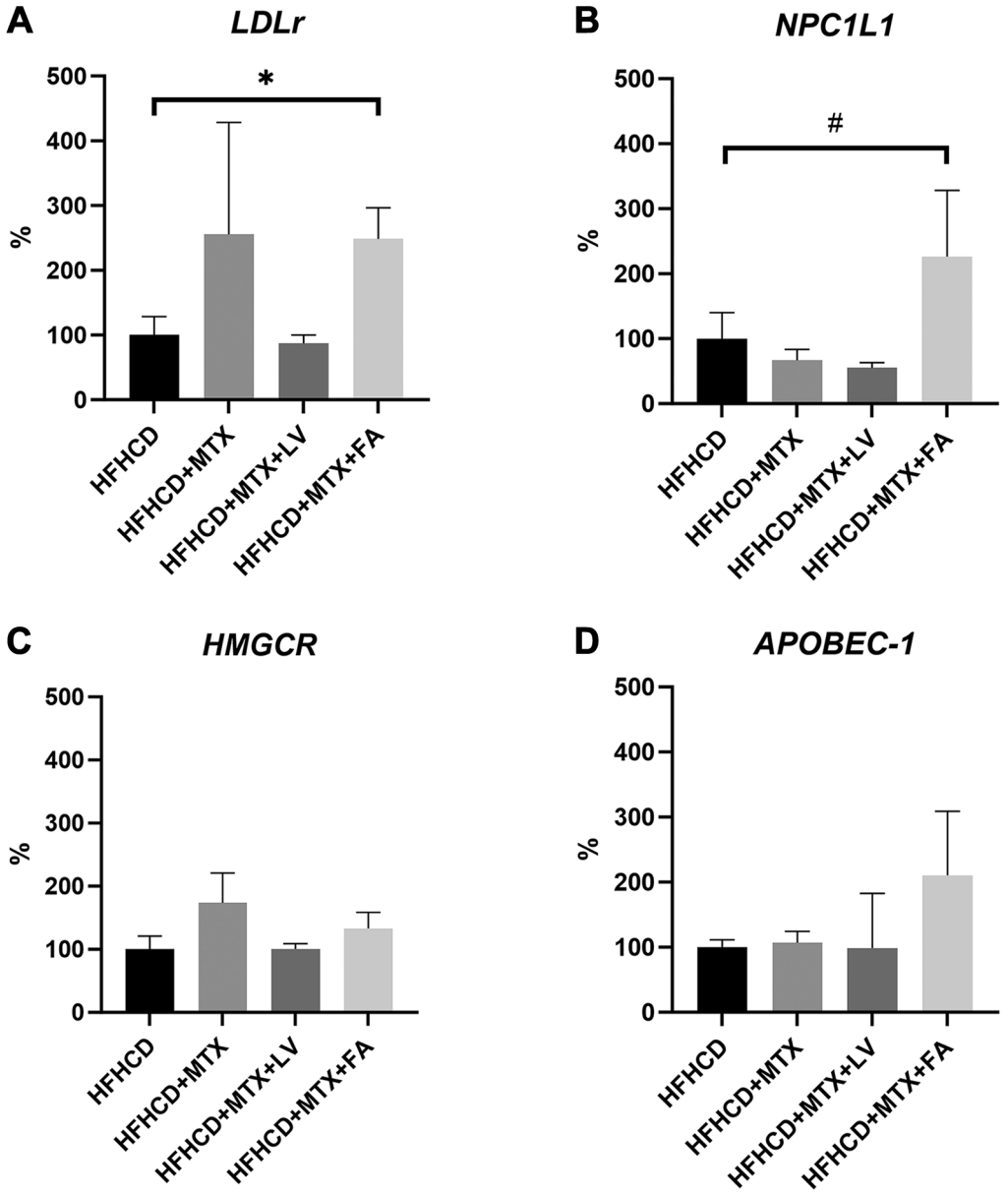

Figure 6. *Expression of hepatic LDLr, HMGCR, NPC1L1, and jejunal APOBEC-1 mRNA. (A) Increased LDLr mRNA expression was observed in the HFHCD+MTX+FA group compared with the HFHCD group. (B) The expression of NPC1L1 was higher in the HFHCD+MTX+FA group than in the HFHCD group. Expressions of hepatic LDLr (C) and jejunal APOBEC-1 (D): No difference was found among the 4 groups. *p<0.05, #p<0.1. HFHCD: High-fat and high-cholesterol diet; MTX: methotrexate; LV: leucovorin; FA: folic acid; LDLr: low-density lipoprotein receptor; HMGCR: 3-hydroxy-3-methylglutaryl-coenzyme A reductase; NPC1L1: Niemann-Pick C1-Like 1; APOBEC-1: apolipoprotein B-100 RNA editing catalytic subunit-1.*

and duration of MTX administration. In the present μMP model, the MTX dose and duration (3.3 mg/body/day for 2 weeks) were much lower and shorter, respectively, than in the standard protocol for RA patients (47). Another possibility is that the presence or absence of complications of other diseases like RA might affect the effect of MTX on atherogenesis. As RA and atherosclerosis share the same pathophysiological features as chronic inflammatory

diseases and exacerbation or progression of atherosclerosis may occur in RA patients (48), further clinical and experimental evaluations would be necessary to clarify the effects of MTX on atherosclerosis.

In conclusion, MTX exacerbates hyperlipidemia-induced atherosclerosis *via* impairment of the folate metabolic pathway but independently by enhancement of hyperlipidemia in the μMP model. This animal model is useful for pathological studies of atherosclerosis associated with MTX treatment as well as pharmacological evaluation of *HMGCR* inhibitors (49). Cardio-oncology is an emerging interdisciplinary field dedicated to the early detection and treatment of adverse cardiovascular events associated with anticancer treatment (50). The present study may promote cardio-oncology research by providing a suitable animal model.

Conflicts of Interest

The Authors declare that no conflicts of interest exist.

Authors' Contributions

Designed the study: YO, NM, AT, and HK. Performed research: YO and HK. Collected and analyzed data: YO and HK. Wrote the paper: YO, NM, AT, and HK.

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References

1 The top 10 causes of death, World Health Organisation, 2020. Available at: https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death [Last accessed on May 4, 2024]

2 Okamura T, Tsukamoto K, Arai H, Fujioka Y, Ishigaki Y, Koba S, Ohmura H, Shoji T, Yokote K, Yoshida H, Yoshida M, Deguchi J, Dobashi K, Fujiyoshi A, Hamaguchi H, Hara M, Harada-Shiba M, Hirata T, Iida M, Ikeda Y, Ishibashi S, Kanda H, Kihara S, Kitagawa K, Kodama S, Koseki M, Maezawa Y, Masuda D, Miida T, Miyamoto Y, Nishimura R, Node K, Noguchi M, Ohishi M, Saito I, Sawada S, Sone H, Takemoto M, Wakatsuki A, Yanai H: Japan Atherosclerosis Society (JAS) guidelines for prevention of atherosclerotic cardiovascular diseases 2022. J Atheroscler Thromb 31(6): 641-853, 2024. DOI: 10.5551/jat.GL2022

3 Ganguly P, Alam SF: Role of homocysteine in the development of cardiovascular disease. Nutr J 14: 6, 2015. DOI: 10.1186/1475-2891-14-6

4 Sobral AF, Cunha A, Silva V, Gil-Martins E, Silva R, Barbosa DJ: Unveiling the therapeutic potential of folate-dependent one-carbon metabolism in cancer and neurodegeneration. Int J Mol Sci 25(17): 9339, 2024. DOI: 10.3390/ijms25179339

5 Rong H, Huang L, Jin N, Hong J, Hu J, Wang S, Xie Y, Pu J: Elevated homocysteine levels associated with atrial fibrillation and recurrent atrial fibrillation. Int Heart J 61(4): 705-712, 2020. DOI: 10.1536/ihj.20-099

6 Raja V, Aguiar C, Alsayed N, Chibber YS, ElBadawi H, Ezhov M, Hermans MP, Pandey RC, Ray KK, Tokgözoglu L, Zambon A, Berrou JP, Farnier M: Non-HDL-cholesterol in dyslipidemia: Review of the state-of-the-art literature and outlook. Atherosclerosis 383: 117312, 2023. DOI: 10.1016/j.atherosclerosis.2023.117312

7 Becker W, Lyhne N, Pedersen AN, Aro A, Fogelholm M, Phorsdottir I, Alexander J, Anderssen SA, Meltzer HM, Pedersen JI: Nordic Nutrition Recommendations 2004 - integrating nutrition and physical activity. Scand J Nutr 48(4): 178-187, 2004. DOI: 10.1080/11026804100037948

8 Townend J, O'Sullivan J, Wilde JT: Hyperhomocysteinaemia and vascular disease. Blood Rev 12(1): 23-34, 1998. DOI: 10.1016/s0268-960x(98)90027-3

9 Welch GN, Loscalzo J: Homocysteine and atherothrombosis. N Engl J Med 338(15): 1042-1050, 1998. DOI: 10.1056/nejm199804093381507

10 Homocysteine Studies Collaboration: Homocysteine and risk of ischemic heart disease and stroke. JAMA 288(16): 2015, 2002. DOI: 10.1001/jama.288.16.2015

11 Den Heijer M, Lewington S, Clarke R: Homocysteine, MTHFR and risk of venous thrombosis: a meta-analysis of published epidemiological studies. J Thromb Haemost 3(2): 292-299, 2005. DOI: 10.1111/j.1538-7836.2005.01141.x

12 Hwang S, Lee Y, Jang Y, Cho JY, Yoon S, Chung JY: Comprehensive evaluation of OATP- and BCRP-mediated drug–drug interactions of methotrexate using physiologically-based pharmacokinetic modeling. Clin Pharmacol Ther 116(4): 1013-1022, 2024. DOI: 10.1002/cpt.3329

13 Morgan SL, Baggott JE, Lee JY, Alarcón GS: Folic acid supplementation prevents deficient blood folate levels and

hyperhomocysteinemia during longterm, low dose methotrexate therapy for rheumatoid arthritis: implications for cardiovascular disease prevention. J Rheumatol 25(3): 441-446, 1998.
14 Ahmed S, Jacob B, Carsons SE, De Leon J, Reiss AB: Treatment of cardiovascular disease in rheumatoid arthritis: a complex challenge with increased atherosclerotic risk. Pharmaceuticals (Basel) 15(1): 11, 2021. DOI: 10.3390/ph15010011
15 Sun KJ, Liu LL, Hu JH, Chen YY, Xu DY: Methotrexate can prevent cardiovascular events in patients with rheumatoid arthritis: An updated meta-analysis. Medicine (Baltimore) 100(7): e24579, 2021. DOI: 10.1097/MD.0000000000024579
16 Getz GS, Reardon CA: Animal models of atherosclerosis. Arterioscler Thromb Vasc Biol 32(5): 1104-1115, 2012. DOI: 10.1161/ATVBAHA.111.237693
17 Johnston JM, Francis SE, Kiss-Toth E: Experimental models of murine atherosclerosis: does perception match reality? Cardiovasc Res 114(14): 1845-1847, 2018. DOI: 10.1093/cvr/cvy140
18 Bilheimer DW, Watanabe Y, Kita T: Impaired receptor-mediated catabolism of low density lipoprotein in the WHHL rabbit, an animal model of familial hypercholesterolemia. Proc Natl Acad Sci U.S.A. 79(10): 3305-3309, 1982. DOI: 10.1073/pnas.79.10.3305
19 Brousseau ME, Hoeg JM: Transgenic rabbits as models for atherosclerosis research. J Lipid Res 40: 365-375, 1999.
20 Fan J, Watanabe T: Transgenic rabbits as therapeutic protein bioreactors and human disease models. Pharmacol Ther 99(3): 261-282, 2003. DOI: 10.1016/s0163-7258(03)00069-x
21 Forster R, Ancian P, Fredholm M, Simianer H, Whitelaw B: The minipig as a platform for new technologies in toxicology. J Pharmacol Toxicol Methods 62(3): 227-235, 2010. DOI: 10.1016/j.vascn.2010.05.007
22 Kawaguchi H, Miyoshi N, Miura N, Fujiki M, Horiuchi M, Izumi Y, Miyajima H, Nagata R, Misumi K, Takeuchi T, Tanimoto A, Yoshida H: Microminipig, a non-rodent experimental animal optimized for life science research: novel atherosclerosis model induced by high fat and cholesterol diet. J Pharmacol Sci 115(2): 115-121, 2011. DOI: 10.1254/jphs.10r17fm
23 Kawaguchi H, Yamada T, Miura N, Ayaori M, Uto-Kondo H, Ikegawa M, Noguchi M, Wang KY, Izumi H, Tanimoto A: Rapid development of atherosclerosis in the world's smallest microminipig fed a high-fat/high-cholesterol diet. J Atheroscler Thromb 21(3): 186-203, 2014. DOI: 10.5551/jat.21246
24 Otake M, Kawaguchi H, Enya S, Kangawa A, Koga T, Matsuo K, Yamada S, Rahman MM, Miura N, Shibata M, Tanimoto A: High pathological reproducibility of diet-induced atherosclerosis in microminipigs *via* cloning technology. In Vivo 35(4): 2025-2033, 2021. DOI: 10.21873/invivo.12471
25 Miyoshi N, Horiuchi M, Inokuchi Y, Miyamoto Y, Miura N, Tokunaga S, Fujiki M, Izumi Y, Miyajima H, Nagata R, Misumi K, Takeuchi T, Tanimoto A, Yasuda N, Yoshida H, Kawaguchi H: Novel microminipig model of atherosclerosis by high fat and high cholesterol diet, established in Japan. In Vivo 24: 671-680, 2010.
26 Bălănescu AR, Bojincă VC, Bojincă M, Donisan T, Bălănescu SM: Cardiovascular effects of methotrexate in immune-mediated inflammatory diseases. Exp Ther Med 17(2): 1024-1029, 2019. DOI: 10.3892/etm.2018.6992
27 Kawaguchi H, Yamada T, Miura N, Takahashi Y, Yoshikawa T, Izumi H, Kawarasaki T, Miyoshi N, Tanimoto A: Reference values of hematological and biochemical parameters for the world smallest microminipigs. J Vet Med Sci 74(7): 933-936, 2012. DOI: 10.1292/jvms.11-0571
28 Kawaguchi H, Yamada T, Miura N, Noguchi M, Izumi H, Miyoshi N, Tanimoto A: Sex differences of serum lipid profile in novel microminipigs. In Vivo 27: 617-621, 2013.
29 Goodrich JA, Clarkson TB, Cline JM, Jenkins AJ, Del Signore MJ: Value of the micropig model of menopause in the assessment of benefits and risks of postmenopausal therapies for cardiovascular and reproductive tissues. Fertil Steril 79 Suppl 79: 779-788, 2003. DOI: 10.1016/s0015-0282(02)04826-4
30 Turk JR, Henderson KK, Vanvickle GD, Watkins J, Laughlin MH: Arterial endothelial function in a porcine model of early stage atherosclerotic vascular disease. Int J Exp Pathol 86(5): 335-345, 2005. DOI: 10.1111/j.0959-9673.2005.00446.x
31 Takai H, Miyoshi A, Yamazaki M, Adachi K, Katagiri K, Arakawa H, Katsuyama K, Ito T, Fujii E, Hayashi S, Kato A, Suzuki M: Granulocyte colony-stimulating factor has no adverse effects on atherosclerotic lesions in high cholesterol-fed miniature swine. J Vet Med Sci 70(9): 943-950, 2008. DOI: 10.1292/jvms.70.943
32 Herrmann J, Saguner AM, Versari D, Peterson TE, Chade A, Olson M, Lerman LO, Lerman A: Chronic proteasome inhibition contributes to coronary atherosclerosis. Circ Res 101(9): 865-874, 2007. DOI: 10.1161/circresaha.107.152959
33 McCully KS: Vascular pathology of homocysteinemia: Implications for the pathogenesis of arteriosclerosis. Am J Pathol 56: 111-128, 1969.
34 Brown MS, Goldstein JL: A receptor-mediated pathway for cholesterol homeostasis. Science 232(4746): 34-47, 1986. DOI: 10.1126/science.3513311
35 Goedeke L, Fernández-Hernando C: Regulation of cholesterol homeostasis. Cell Mol Life Sci 69(6): 915-930, 2012. DOI: 10.1007/s00018-011-0857-5
36 Albert CM, Cook NR, Gaziano JM, Zaharris E, MacFadyen J, Danielson E, Buring JE, Manson JE: Effect of folic acid and B vitamins on risk of cardiovascular events and total mortality among women at high risk for cardiovascular disease: a randomized trial. JAMA 299(17): 2027-2036, 2008. DOI: 10.1001/jama.299.17.2027
37 Løland KH, Bleie O, Borgeraas H, Strand E, Ueland PM, Svardal A, Nordrehaug JE, Nygård O: The association between progression of atherosclerosis and the methylated amino acids asymmetric dimethylarginine and trimethyllysine. PLoS One 8(5): e64774, 2013. DOI: 10.1371/journal.pone.0064774

38 Kataria N, Yadav P, Kumar R, Kumar N, Singh M, Kant R, Kalyani V: Effect of vitamin B6, B9, and B12 supplementation on homocysteine level and cardiovascular outcomes in stroke patients: a meta-analysis of randomized controlled trials. Cureus 13(5): e14958, 2021. DOI: 10.7759/cureus.14958
39 Hidayat R, Fauzia F, Parlindungan F, Wibowo SAK, Ariane A, Damanik J, Araminta AP: Predictive factors of methotrexate monotherapy success in patients with rheumatoid arthritis in a national referral center: a cohort study. BMC Rheumatol 8(1): 42, 2024. DOI: 10.1186/s41927-024-00412-8
40 van Ede AE, Laan RF, Blom HJ, Boers GH, Haagsma CJ, Thomas CM, De Boo TM, van de Putte LB: Homocysteine and folate status in methotrexate-treated patients with rheumatoid arthritis. Rheumatology (Oxford) 41(6): 658-665, 2002. DOI: 10.1093/rheumatology/41.6.658
41 van Breukelen-van der Stoep DF, Klop B, van Zeben D, Hazes JM, Castro Cabezas M: Cardiovascular risk in rheumatoid arthritis: How to lower the risk? Atherosclerosis 231(1): 163-172, 2013. DOI: 10.1016/j.atherosclerosis.2013.09.006
42 Hjeltnes G, Hollan I, Førre O, Wiik A, Lyberg T, Mikkelsen K, Agewall S: Serum levels of lipoprotein(a) and E-selectin are reduced in rheumatoid arthritis patients treated with methotrexate or methotrexate in combination with TNF-alpha-inhibitor. Clin Exp Rheumatol 31(3): 415-421, 2013.
43 Deyab G, Hokstad I, Whist JE, Smastuen MC, Agewall S, Lyberg T, Ronda N, Mikkelsen K, Hjeltnes G, Hollan I: Methotrexate and anti-tumor necrosis factor treatment improves endothelial function in patients with inflammatory arthritis. Arthritis Res Ther 19(1): 232, 2017. DOI: 10.1186/s13075-017-1439-1
44 Avagimyan A, Fogacci F, Pogosova N, Kakturskiy L, Jndoyan Z, Faggiano A, Bairamyan T, Agati L, Sattar Y, Mkrchyan L, Avetisyan G, Ginosyan K, Aznauryan A, Sahakyan K, Trofimenko A, Urazova O, Mikhaleva L, Vandysheva R, Kogan E, Demura T, Kc M, Shafie D, Nicola S, Brussino L, Cicero A, Biondi-Zoccai G, Sarrafzadegan N: Methotrexate & rheumatoid arthritis associated atherosclerosis: A narrative review of multidisciplinary approach for risk modification by the international board of experts. Curr Probl Cardiol 49(2): 102230, 2024. DOI: 10.1016/j.cpcardiol.2023.102230
45 Johnson TM, Sayles HR, Baker JF, George MD, Roul P, Zheng C, Sauer B, Liao KP, Anderson DR, Mikuls TR, England BR: Investigating changes in disease activity as a mediator of cardiovascular risk reduction with methotrexate use in rheumatoid arthritis. Ann Rheum Dis 80(11): 1385-1392, 2021. DOI: 10.1136/annrheumdis-2021-220125
46 Golledge J, Velu R, Quigley F, Jenkins J, Singh TP: Cohort study examining the association of immunosuppressant drug prescription with major adverse cardiovascular and limb events in patients with peripheral artery disease. Ann Vasc Surg 78: 310-320, 2022. DOI: 10.1016/j.avsg.2021.07.010
47 Manaï M, van Middendorp H, Veldhuijzen DS, van der Pol JA, Huizinga TWJ, Evers AWM: Pharmacological conditioning in the treatment of recent-onset rheumatoid arthritis: a randomized controlled trial study protocol. Trials 21(1): 15, 2020. DOI: 10.1186/s13063-019-3777-6
48 Kerola AM, Rollefstad S, Semb AG: Atherosclerotic cardiovascular disease in rheumatoid arthritis: impact of inflammation and antirheumatic treatment. Eur Cardiol 16: e18, 2021. DOI: 10.15420/ecr.2020.44
49 Yamada T, Kawaguchi H, Matsuoka A, Akioka K, Miura N, Izumi H, Tanimoto A: Development of a microminipig model of atherosclerosis for the evaluation of a HMGCR inhibitor. In Vivo 38(1): 98-106, 2024. DOI: 10.21873/invivo.13415
50 Kuang Z, Kong M, Yan N, Ma X, Wu M, Li J: Precision cardio-oncology: update on omics-based diagnostic methods. Curr Treat Options Oncol 25(5): 679-701, 2024. DOI: 10.1007/s11864-024-01203-6