Newly identified antiatherosclerotic activity of methotrexate and adalimumab: Complementary effects on lipoprotein function and macrophage cholesterol metabolism

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**Running head:** Lipid-related anti-atherosclerotic effects of methotrexate and adalimumab.

**Title:** New anti-atherosclerotic activity of methotrexate and adalimumab: complementary effects on lipoprotein function and macrophage cholesterol metabolism.

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**Abstract**

**Objective**: Rheumatoid arthritis (RA) is associated with accelerated atherosclerosis. The reduction of cardiovascular risk induced by methotrexate and anti-TNFα agents in RA is considered secondary to their anti-inflammatory action, but their effects on serum lipoprotein function and foam cell formation are unknown. The reduced high density lipoprotein (HDL) capacity to promote cell cholesterol efflux (CEC) and the increased serum cell cholesterol-loading capacity (CLC) demonstrated in RA may contribute to foam cell development. We investigated the influence of methotrexate and adalimumab treatment on serum CEC and CLC in RA patients. Furthermore, we studied the *in vitro* effect of the two drugs on macrophage cholesterol handling.

**Methods**: serum from RA patients treated with methotrexate (n=34) or with adalimumab+methotrexate (n=22), drawn before and after 6 weeks and 6 months of treatment, was analyzed for CEC and CLC by radioisotopic and fluorimetric techniques, respectively. Methotrexate and adalimumab influence on macrophage cholesterol efflux and uptake was evaluated *in vitro* on human THP-1-derived macrophages.

**Results**: methotrexate treatment was associated with serum HDL, LDL and total cholesterol increase, with ATP-binding cassette G1 and Scavenger receptor-BI (SR-BI)-mediated CEC increase and no CLC modification. Adalimumab treatment was associated with serum HDL increase, SR-BI-mediated and ATP-binding cassette A1-mediated CEC transient increase and decrease respectively, and with a significant CLC reduction; in addition, adalimumab reduced macrophage cholesterol uptake *in vitro*.

**Conclusion**: methotrexate and adalimumab anti-atherosclerotic activity may be mediated by beneficial and complementary effects on lipoprotein functions and on macrophage cholesterol handling, as a whole opposing foam cell formation.

**Keywords**: rheumatoid arthritis, methotrexate, adalimumab, atherosclerosis, cholesterol.

Rheumatoid Arthritis (RA) is associated with accelerated atherosclerosis that significantly contributes to the mortality excess (1,2). Among the mechanisms of vascular damage in RA are inflammation and autoimmune processes, but also lipid metabolism disturbances differing from classical pro-atherogenic dyslipidaemia (3). Specifically, modifications in the composition and function of lipoproteins have been reported. In patients with RA, high density lipoproteins (HDL) have reduced anti-inflammatory and anti-oxidant effects (4,5) and impaired capacity to promote cell cholesterol efflux (6). Cholesterol efflux is the first step of reverse cholesterol transport, a process leading to the biliary excretion of cholesterol in the intestine (7). Macrophage cholesterol efflux occurs through aqueous diffusion (AD), but especially through the activity of the membrane transporters Scavenger Receptor class B type I (SR-BI), ATP Binding Cassette A1 (ABCA1) and ATP Binding Cassette G1 (ABCG1). In all cases the presence of extracellular acceptors, mainly constituted by HDL, is necessary for cholesterol efflux to occur (8,9). During the generation and remodelling of HDL, various subclasses develop, with differential and specific activity with respect to single cholesterol transporters. Serum cholesterol efflux capacity (CEC) is the measure of total or transporter-specific activity of circulating cholesterol acceptors, mainly HDL, through standardized methods (10). This parameter has been demonstrated to correlate directly with vascular functionality and inversely with atherosclerosis (11–14). The relevance of a functional parameter such ***as*** CEC in autoimmune and inflammatory diseases is underlined by the evidence that cell cholesterol efflux, besides acting as a mechanism limiting intracellular cholesterol content, is coupled with regulatory intracellular signalling; the resulting modulation of several cell functions, both in macrophages and endothelial cells, leads to the inhibition of inflammatory and immune reactions (15,16). These events could be of particular interest in RA patients, as the presence of foam cells has also been described in synovial tissue (17).

In addition, emerging data indicate that the macrophage cholesterol loading capacity (CLC) of serum is increased in RA patients (18), possibly due to modifications in the composition and physical-chemical properties of low density lipoproteins (LDL), which are the main cell cholesterol donor, and/or to the presence of autoantibodies (19).

Methotrexate (MTX) and anti-TNFα agents reduce cardiovascular risk in RA patients with mechanisms that are at present unknown and generically attributed to their anti-inflammatory action (20–23). These agents would instead have unfavourable (MTX) effects or no effect (anti-TNFα agents) on serum lipid levels (24,25). However, given both the relevance of functional properties of circulating lipoproteins along with their serum levels with respect to CV risk (26,27), and the lack of data on the possible effects of MTX and adalimumab (ADA) on lipoprotein function and macrophage cholesterol trafficking, we evaluated CLC and CEC in RA patients treated with MTX or ADA in association with MTX and studied the direct effects of these drugs on macrophage cholesterol uptake and efflux.

## MATERIALS AND METHODS

**Patients.** We selected 56 patients with active RA, starting with either MTX or ADA, from the Norwegian observational PSARA study (28). Diagnosis was confirmed according to the American Rheumatism Association diagnostic criteria for RA (29). Written informed consent was obtained from each patient and approval from the local ethical committee was granted (PSARA registered clinical trial n° NCT00902005). Treatment prescription was based on clinical indication and followed standard European/Norwegian rules. Therefore, all patients starting ADA had a history of previously failed MTX treatment. To minimize side effects, ADA was used in combination with MTX (except for one patient who was intolerant to MTX). For each patient, clinical and laboratory data together with serum samples were obtained before therapy, after 6 weeks and after 6 months of therapy with MTX (MTX, n = 34) or with ADA in combination with MTX (ADA-MTX, n = 22). Patients' main characteristics are summarised in Table 1S (supplementary material).

**Serum cholesterol efflux capacity (CEC).**

Serum CEC was measured in samples drawn before treatment and after 6 weeks and 6 months of treatment with MTX or with ADA-MTX, using cell-based standardized methods (10). Specific pathways of cholesterol efflux were studied using adequate cell models: J774 macrophages incubated with or without cAMP (Sigma Aldrich, Milano, Italy) for the evaluation of ABCA1- and AD-mediated efflux respectively; rat hepatoma Fu5AH in the absence or presence of a specific SR-BI inhibitor (Block Lipid Transfer-1 10μM, purchased from ChemBridge, San Diego, CA) for SR-BI-cholesterol efflux; Chinese hamster ovary cells transfected or not with the human *abcg1* gene for ABCG1-mediated efflux. In all assays the cells were labelled with [1,2-$^{3}$H]-cholesterol (PerkinElmer, Milano, Italy) for 24 hours, incubated with 0.2% bovine serum albumin in medium for 24 hours and treated for 4 or 6 hours with 1% or 2% (v/v) whole serum, depending on the cholesterol efflux pathway to be

analysed. Serum CEC results were expressed as a percentage of total radioactivity incorporated by the cells that was released in the supernatant. A pool of human normolipidaemic sera was tested in each assay as reference standard 1 and its CEC was used to normalize the patient samples values from the different experiments, in order to correct for the inter-assay variability. A second pool of human normolipidaemic sera as reference standard 2 was tested in each assay and its CEC, after normalization, was the index of the intra-assay variability (10).

**Serum cholesterol loading capacity (CLC).**

THP-1 cells were cultured in 12-well plates in the presence of 50 ng/ml Phorbol 12-Myristate 13-Acetate (PMA) (Sigma Aldrich, Milano, Italy) for 72 hours to allow differentiation into macrophages. Cells were exposed for 24 hours to serum collected from patients with RA before therapy and after 6 weeks or 6 months of therapy with MTX or with ADA-MTX. Serum dilution in this model is 5%. To exclude the influence of LDL levels, we calculated each serum dilution in order to normalize LDL-C concentration to 120 mg/dl. Cell cholesterol content was measured by fluorimetric detection in cell extracts (30). An aliquot of the cell lysates was taken to measure cell protein by a modified Lowry method (31). CLC was defined as macrophage cholesterol content in the cell extract after exposure of cells to serum and expressed as micrograms of cholesterol per milligram of protein.

***In vitro* interaction between living macrophages and ADA.**

Before performing the *in vitro* study on the direct effect of ADA (purchased as commercial drug, Abbott Laboratories, UK), on macrophage cholesterol influx and efflux, we demonstrated the actual interaction between ADA and living THP-1 derived macrophages in our experimental conditions. THP-1 cells were cultured in an 8-well multi-chamber glass slide in the presence of 50 ng/ml PMA for 72 hours to allow differentiation into macrophages.

Living cells were pre-treated with LPS 1 μg/ml for 2 hours to induce TNFα membrane expression and then incubated with ADA 15 μg/ml for 15 or 50 minutes. Cells were then washed and fixed for 2 minutes with methanol at 4°C. After washing, the cells were incubated with a secondary anti-human IgG FITC-conjugated antibody (Life Technologies, Monza, Italy) for 40 minutes. Cells were washed and observed using a LSM 510 META Zeiss confocal microscope, with an X63 oil objective. As a control IgG isotype antibody we used 15 μg/ml rituximab (purchased as commercial drug, Roche, UK).

***In vitro* effect of MTX and ADA on macrophage cholesterol uptake.**

THP-1 cells were cultured in 12-well plates in presence of 50 ng/ml PMA for 72 hours to allow differentiation into macrophages. In experiments with ADA, cells were pre-treated with Lipopolysaccharide (LPS) (Sigma Aldrich, Milano, Italy) 1 μg/ml for 2 hours to induce TNFα membrane expression. MTX (purchased as commercial drug, Methotrexate injectable solution, Sandoz Spa, Varese, Italy) was used at 0.25 μM/L and ADA at 15 μg/ml, concentrations corresponding to the mean circulating levels of the two molecules during chronic therapy (32–34). As a control IgG isotype antibody for adalimumab we used 15 μg/ml rituximab. After 18 or 2 hour treatment with either MTX or ADA respectively, cells were incubated for 24 hours with 5% normal human serum as cholesterol donor in the presence of the respective drug. The cholesterol content of the cells was measured as described for serum CLC in the previous paragraph.

***In vitro* effect of MTX and ADA on macrophage cholesterol efflux.**

THP-1 cells were cultured in 12-well plates in the presence of 50 ng/ml PMA for 72 hours to allow differentiation into macrophages. Cells were radiolabeled for 24h with [1,2- $^{3}$H] cholesterol in presence of Acyl-CoA:Cholesterol Acyltransferase inhibitor (Sigma Aldrich,

Milano, Italy) 2 μg/ml to maintain cholesterol in free, not esterified form. In experiments with ADA, cells were pre-treated with LPS 1 μg/ml for 2 hours to induce TNFα membrane expression. MTX was used at 0.25 μM/L and ADA at 15 μg/ml, concentrations corresponding to the mean circulating levels of the two molecules during chronic therapy (32–34). After 18 hour incubation with either MTX or ADA, cholesterol efflux was induced by incubation of cells with 2% normal human serum for 8 hours. Cholesterol efflux was expressed as the percentage of total radioactivity incorporated by the cells that was released in the supernatant. Similar experiments were performed pre-incubating the cells for 24 hours with acetylated LDL 50 mg/dl to load cells with cholesterol.

**Statistical analysis**

Statistical analyses were performed using Prism (version 5.0) (GraphPad Inc., San Diego, CA). Each experiment was run in triplicate. Data were expressed as mean ± SD if not indicated otherwise. Specific pathway-mediated CEC and CLC values at the various time points were compared within each treatment group using the Student's t test for paired samples. The relationships between specific pathway-mediated CEC values and other clinical and laboratory parameters were evaluated by linear regression analysis. The values of cholesterol influx and efflux in macrophages in basal conditions and after treatment with MTX or ADA were compared using the Student's t test for unpaired samples.

## RESULTS

### Serum lipid levels in patients treated with MTX or with ADA-MTX.

As shown in Table 1, total cholesterol (TC), LDL and HDL were increased after 6 months of therapy with MTX alone. A slight increase in HDL levels was observed in the ADA-MTX group after 6 weeks and after 6 months; statistical significance was reached only at 6 weeks. No statistically significant differences in TC/HDL ratio between time points within each group of treatment were found, except for the 6 weeks value in the MTX group, which was reduced as compared to the baseline (3.82 at 6 weeks, 4.04 at baseline, $p < 0.01$, paired Student's t test).

### Effect of MTX or ADA-MTX treatment on serum CEC.

In patients treated with MTX alone, SR-BI-mediated serum CEC was significantly increased after 6 weeks and 6 months of treatment as compared to the baseline (Figure 1A), ABCA1-mediated CEC was unchanged (Figure 1B) and ABCG1-mediated CEC was increased after 6 months of therapy (Figure 1C). ADA-MTX treatment was associated with a transient increase of SR-BI-mediated CEC (Figure 1D) and a transient decrease of ABCA1-mediated CEC (Figure 1E) after 6 weeks, with no modifications in ABCG1-mediated CEC (Figure 1F).

### Relationship between serum CEC and total circulating HDL levels.

HDL are the major physiologic cholesterol acceptors in cell cholesterol efflux processes. Serum SR-BI-mediated CEC correlated with HDL circulating levels in both treatment groups, before and after treatment, and ABCA1-mediated CEC on no occasion correlated with serum HDL levels, similar to that observed in the healthy population (6). The positive correlation usually found in healthy subjects between ABCG1-mediated CEC and total serum HDL (6) was absent in the MTX group for all time points and in the ADA-MTX group before and after

6 weeks of therapy; however, it was restored after 6 months of ADA-MTX treatment (Table 2).

**Relationship between serum CEC and disease activity score.**

No significant relationship was observed between SR-BI and ABCA1-mediated CEC and the disease activity score DAS28 (calculated with erythrocyte sedimentation rate). A significant inverse relationship between serum ABCG1-mediated CEC and DAS28 was detected in the ADA-MTX group only, after 6 months of treatment (Table 2).

**Relationship between serum CEC and homocysteine**

No significant relationship was observed between SR-BI- and ABCA1-mediated CEC and serum homocysteine levels. An inverse relationship, very close to statistical significance, between serum ABCG1-mediated CEC and homocysteine levels was found after both 6 weeks and 6 months of treatment in the ADA-MTX group only (Table 2). Pooling the data relative to post-treatment sera, a significant inverse relationship between ABCG1-mediated CEC and homocysteine was found in the ADA-MTX group but not in the MTX group (Table 2).

**Relationship between serum CEC and inflammatory indexes.**

We did not find any significant relationship between specific transporter-mediated CEC and serum inflammatory indexes such as ESR, CRP, TNFα, or adhesion molecules, at any time point nor for any of the treatment regimens (data not shown).

**Effect of MTX or ADA-MTX treatment on serum CLC.**

Serum CLC was measured in samples obtained before and after 6 months of treatment. CLC was not significantly modified after treatment with MTX (p= 0.46) (Figure 2, left panel), but

was significantly reduced after ADA-MTX treatment ($p<0.05$) (Figure 2, right panel). To verify whether this effect was dependent on the blockade of soluble TNFα in serum, we incubated THP-1 derived macrophages with human recombinant TNFα (Sigma Aldrich, Milano, Italy) and measured cell cholesterol uptake. We found that TNFα did not influence cholesterol uptake in our in vitro system (Figure 1S, supplementary material). Moreover, serum CLC values did not correlate with TNFα circulating levels (data not shown).

***In vitro* effects of MTX and ADA on macrophage cholesterol handling.**

The incubation of THP-1 derived macrophages with MTX 0.25 μM/L, the reported mean circulating concentration during chronic therapy (32), did not influence cholesterol uptake nor cholesterol efflux (Figure 3).

ADA has been reported to bind to TNFα expressed on the cell membrane and to induce a so-called "reverse signaling" (35,36). We thus: a) evaluated whether our model was suitable to investigate the possible consequences of ADA interaction with macrophage membrane on cholesterol trafficking, performing confocal microscopy studies to document the actual interaction of ADA with living THP-1 derived macrophages; b) measured the effect of ADA on cholesterol influx and efflux. ADA at 15 μg/ml, the reported mean circulating concentration during chronic therapy (33,34), bound to and was internalised by living THP-1 derived macrophages, pre-treated with LPS in order to induce membrane TNFα expression (Figure 4, panels A and B). Under the same conditions, ADA inhibited THP-1 derived macrophages cholesterol uptake from both normal (cell cholesterol content 6.63±0.92 vs 10.58±0.36 μg cholesterol/mg protein, $p<0.01$) and hypercholesterolaemic serum (cell cholesterol content 5.3 ±0.55 vs 11.8 ±0.19 μg cholesterol/mg protein, $p<0.0001$) (Figure 4, panel C). The incubation of THP-1 derived macrophages with human recombinant TNFα (Sigma Aldrich, Milano, Italy) did not influence cholesterol uptake (Figure 1S, supplementary

material). Under the same experimental conditions used for adalimumab, the IgG isotype control antibody rituximab showed no binding to THP-1 derived macrophages (immunofluorescence negative data are not shown because consisting in dark microscopic fields) nor any effect on cell cholesterol loading (Figure 2S, supplementary material).

ADA treatment did not influence cholesterol efflux (Figure 4, panel D). The same result was obtained in cells pre-loaded with cholesterol accomplished through overnight incubation with acetylated LDL at 50 mg/dl (data not shown).

## DISCUSSION

The results of this novel study indicate that the reduction of cardiovascular risk induced in RA patients by MTX and ADA treatment (20–23) might be due not only to the repression of inflammation but also to specific anti-atherogenic effects of these drugs, mediated by a direct influence on lipoprotein functions and macrophage cholesterol handling.

Serum cholesterol efflux capacity (CEC) is measured through standardized techniques and reflects mainly the ability of HDL to accept cholesterol from macrophages. This HDL functional parameter is correlated directly with indices of vessel health (11,12) and inversely with cardiovascular risk (13,14). MTX treatment is associated with an improvement in serum CEC mediated by the cholesterol transporters SR-BI and ABCG1. Within serum HDL sub-fractions, the particles specifically accepting cholesterol from SR-BI constitute the majority of circulating HDL, so it is possible that the MTX-induced SR-BI-mediated CEC improvement is related to the increase in total serum HDL. Indeed, we found a positive significant relationship between the two parameters. Our observation on the parallel increase in serum total HDL and SR-BI-mediated CEC is not trivial because certain spontaneous (12) or pharmacologically-induced conditions (37) characterized by high serum HDL are associated with the production of non-functional HDL.

The relevance of SR-BI- and ABCG1-mediated CEC increase induced by MTX relies on the fact that macrophage cholesterol efflux not only opposes foam cell formation, but is also associated with intracellular anti-inflammatory signals in macrophages and endothelial cells (15). The cardiovascular protective activity of MTX (20) might thus be, at least in part, the consequence of a direct effect of the drug on lipoprotein functions. This adds complementarily to the reported capacity of MTX to increase the expression of *abca1* gene in peripheral blood mononuclear cells (38).

The combination treatment with ADA and MTX induces no substantial modification in serum CEC, suggesting that ADA does not influence CEC, except for a transient increase and

decrease in SR-BI and ABCA1-mediated CEC, respectively. Such variations could be due to the temporary activation of enzymes involved in HDL remodelling, such as Lecithin cholesterol Acyl Transferase (LCAT), causing a switch from small immature HDL, specific for the ABCA1 transporter, to larger and more mature HDL, specific for SR-BI (39–41). Interestingly, an impairment of LCAT activity in RA patients has been reported (42). It is possible to speculate that anti-TNFα agents might anyway improve reverse cholesterol transport *in vivo*, due to their efficacy in counteracting TNFα inhibition of the expression of genes involved in cholesterol efflux, as demonstrated for infliximab (43).

ADA-MTX treatment, and not MTX alone, is specifically associated with modifications in the relationship of ABCG1-mediated CEC with total HDL levels, disease activity and the cardiovascular risk marker homocysteine. We have previously reported that ABCG1-mediated CEC was impaired and inversely correlated to disease activity in an Italian cohort of RA patients (6). Moreover, in the same population, the physiological direct relationship between ABCG1-mediated CEC and total HDL levels (6,42,44,45) was lost, indicating that in RA the production of the HDL sub-fraction specific for ABCG1 is not proportional to that of the total HDL. In the present study we found that ADA restored the inverse relationship between ABCG1-mediated CEC and DAS28 and the direct relationship with total HDL, pointing to a distinctive effect of ADA in regulating the ABCG1-specific HDL sub-fraction. At present it is difficult to speculate on the possible underlying mechanisms, but this concept is also supported by the finding that ADA induced the appearance of an inverse relationship between serum ABCG1-mediated CEC and the cardiovascular risk marker homocysteine. The mechanisms by which ADA unmasks the connection between homocysteine levels and HDL dysfunction, possibly related to oxidative stress, are unknown and deserve further investigation, but it is interesting that ADA again displayed a distinctive ability to influence the ABCG1-specific HDL fraction, one not shared by MTX.

Overall, confirming our own (6) and other (46) previous reports, we did not find any correlation between specific transporter-mediated CEC and serum inflammatory indexes (such as ESR, CRP, TNFα, or adhesion molecules), at any time point nor for any of the treatment regimens. This indicates that the role of inflammation on HDL capacity to promote cell cholesterol efflux, if any, is complex and not linked to any single parameter.

The significant reduction in CLC observed in patients treated with ADA, independent of LDL serum levels, might be a new anti-atherogenic mechanism by which this treatment contributes to cardiovascular protection in RA patients, especially considering that patients in the ADA-MTX group presented higher CLC values at baseline with respect to MTX treatment group. Serum CLC depends mainly on LDL (30) and can be influenced by LDL composition and physical-chemical properties, but also by other circulating agents such as autoantibodies (19). ADA action on one or more of these factors could thus oppose foam cell formation.

On the other hand, ADA does not seem to reduce CLC though the blockade of serum TNFα, because exogenous TNFα in our model did not induce an increase in cell cholesterol loading.

In addition, ADA opposes macrophage cholesterol accumulation through another and possibly complementary effect, *i.e.* the direct inhibition of cholesterol uptake at the macrophage level. ADA has been reported to bind to TNFα expressed on the cell membrane and to induce a so-called "reverse signaling". We demonstrated that ADA in our model, at concentrations usually found in patients undergoing chronic therapy, is able to interact with living macrophages stimulated to express membrane TNFα, and to reduce cell cholesterol content after incubation with normal serum used as cholesterol donor. The actual existence of two additive mechanisms for ADA inhibition of macrophage cholesterol accumulation is supported by the fact that our serum CLC assay is performed using high serum dilution, so that the amount of ADA which gets in direct contact with the cells is too small to affect their

function. The direct ADA action would be particularly relevant on macrophages expressing membrane TNFα, as in RA patients with active disease and in atherosclerotic plaques (36).

In our studies MTX did not modify resting macrophage cholesterol uptake nor efflux *in vitro*, but positive direct effects on cholesterol efflux transporters and on foam cell formation was demonstrated in cytokine-activated cells (47).

The serologic study has some limitations. According to the current indications anti-TNFα agents can be initiated only after an unsuccessful trial with MTX, so the patients in the two populations differ for disease duration and history. To minimize the effect of this confounding factor, we did not compare data from the two patient groups but rather took into account the variations in CEC and CLC with treatment compared to the baseline within each group. Another limitation is that this is an observational study and not a randomised controlled trial; however, as such, it gives a picture of a real-life population. Finally, the small number of patients limited the potential for detailed statistical analyses. Still, the study was large enough to demonstrate several valuable differences and relationships. As this is the first study to evaluate the effect of MTX and ADA-MTX combination therapy on lipoprotein functions and macrophage cholesterol handling, it gives important direction for further research.

In summary, in the present study we demonstrated for the first time that MTX and ADA might exert an athero-protective action through the regulation of cholesterol metabolism, which can be partially independent of their anti-inflammatory activity. This is consistent with very recent data indicating that MTX and anti-TNFα agent therapies are able to disconnect rapid intima-media thickness progression from erythrocyte sedimentation rate (48). The athero-protective effects of MTX and ADA, both with positive effects on foam cell formation, are possibly complementary as they differ with respect to the mechanisms, in that MTX

improves HDL capacity to promote cell cholesterol efflux and ADA reduces serum CLC and macrophage cholesterol uptake.

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## FIGURE LEGEND

**Figure 1.** Serum SR-BI-, ABCA1-, ABCG1-mediated CEC before treatment and after 6 weeks and 6 months of treatment with MTX (panels A-B-C) or ADA-MTX (panels D-E-F). Values are expressed as a percentage of total cell cholesterol that was released in cell supernatants. Statistical significance was calculated by paired Student's t test.

**Figure 2.** Serum CLC at baseline and after 6 months of treatment with MTX (left panel) or ADA-MTX (right panel). Macrophage cholesterol content after incubation with patients' sera is expressed as micrograms per milligram of proteins. Statistical significance was calculated by paired Student's t test.

**Figure 3.** *In vitro* effect of MTX on cholesterol influx and efflux in macrophages. Panel A: The treatment of THP-1 derived macrophages with MTX 0.25 μM/L did not influence cell cholesterol uptake from normal (NHS) and hypercholesterolaemic (HCS) human serum used as cholesterol donors. Panel B: The treatment of THP-1 derived macrophages with MTX 0.25 μM/L did not influence cell cholesterol efflux to normal human serum (NHS) used as cholesterol acceptor. No statistical significance, calculated by unpaired Student's t test, was found between conditions with or without MTX.

**Figure 4.** *In vitro* interaction of ADA with THP-1 derived macrophages and effect on cholesterol influx and efflux. Panels A and B: binding and internalization of ADA; living cells were treated with LPS 1μg/ml for 2 hours and then with ADA 15 μg/ml for 15 minutes (panel A) or 50 minutes (panel B). Cells were then fixed and incubated with an anti-human IgG coupled with fluorescein isothyocyanate. In panel A the signal can be observed mainly on the cell membrane; in panel B the signal on the cell membrane has faded and appears

predominantly as intra-cytoplasmic granules. Bar = 10 μm. Panel C: THP-1 derived macrophages were treated with LPS 1μg/ml and then with ADA 15 μg/ml; ADA significantly reduced cell cholesterol uptake from normal (NHS) and hypercholesterolaemic (HCS) human serum used as cholesterol donors. Panel D: THP-1 derived macrophages were treated with LPS 1μg/ml and then with ADA 15 μg/ml; ADA did not influence cell cholesterol efflux to normal human serum (NHS) used as cholesterol acceptor. Statistical significance was calculated by unpaired Student's t test between conditions with or without ADA. ** = $p < 0.005$ and *** = $p < 0.0001$.

**Figure 1S.** *In vitro* effect of soluble TNFα on macrophage cholesterol uptake. Cells were incubated with medium (basal) or with 20 ng/ml TNFα for 24 hours (TNFα), or exposed to normal human serum for 24 hours with a 4 hour pre-incubation with medium (NHS) or TNFα (TNFα NHS). Macrophage cholesterol content is expressed as micrograms per milligram of proteins. Statistical significance was calculated by paired Student's t test, NS = not significant.

**Figure 2S.** *In vitro* effect of a control IgG isotype antibody for adalimumab on cell cholesterol uptake. THP-1 derived macrophages were treated with LPS 1μg/ml and then with rituximab (RIT) 15 μg/ml. Macrophage cholesterol content is expressed as micrograms per milligram of proteins. Rituximab showed no effect on cholesterol influx in macrophages. Statistical significance was calculated by paired Student's t test, NS = not significant.

**Table 1**.

Lipid profile of patients with RA at baseline and after 6 weeks and 6 months of treatment with MTX and ADA-MTX.

| | **MTX baseline** | **6 weeks** | **6 months** |
|---|---|---|---|
| **TC (mg/dL)** | 216.4 ± 41.8 | 220.2 ± 41.6 | 234.1 ± 50.6* |
| **LDL (mg/dL)** | 128.8 ± 38.1 | 132.5 ± 36.6 | 140.6 ± 41.7* |
| **HDL (mg/dL)** | 53.5 ± 13.9 | 57.71 ± 19.56 | 61.06 ± 20.0** |
| **Triglycerides(mg/dL)** | 114.1 ± 47.8 | 100.8 ± 35.5 | 110.6 ± 50.5 |
| | **ADA-MTX baseline** | **6 weeks** | **6 months** |
| **TC (mg/dL)** | 224.3 ± 47.9 | 233.8 ± 48.7 | 231.3 ± 48.6 |
| **LDL (mg/dL)** | 133.6 ± 40.7 | 139.5 ± 44.9 | 136.7 ± 41.6 |
| **HDL (mg/dL)** | 58.7 ± 17.7 | 64.05 ± 17.6* | 63.7 ± 20.1 |
| **Triglycerides (mg/dL)** | 109.1 ± 30.6 | 107.4 ± 30.8 | 115.2 ± 30.8 |

* $p < 0.05$ and ** $p < 0.001$ vs baseline within each group.

**Table 2.**

Relationship between ABCG1-mediated CEC and serum HDL levels, DAS28 and homocysteine.

| **ABCG1-mediated CEC** | | **baseline** $r^2$ (p) | **6 weeks** $r^2$ (p) | **6 months** $r^2$ (p) |
|---|---|---|---|---|
| Correlation with serum HDL | MTX | 0.006 (0.67) | 0.044 (0.23) | 0.01 (0.572) |
| | ADA-MTX | 0.001 (0.89) | 0.107 (0.14) | **0.18 (0.047)** |
| Correlation with DAS28 | MTX | 0.037 (0.28) | 0.055 (0.18) | 0.010 (0.573) |
| | ADA-MTX | 0.052 (0.31) | 0.003 (0.82) | **0.247 (0.018)** |
| Correlation with homocysteine | MTX | 0.001 (0.89) | 0.001 (0.95) | 0.001 (0.83) |
| | ADA-MTX | 0.001 (0.98) | 0.150 (0.07) | 0.146 (0.07) |
| pooled post treatment | MTX | | 0.001 (0.896) | |
| pooled post treatment | ADA-MTX | | **0.137 (0.013)** | |

In bold are statistically significant relationship values, evaluated by linear regression analysis.

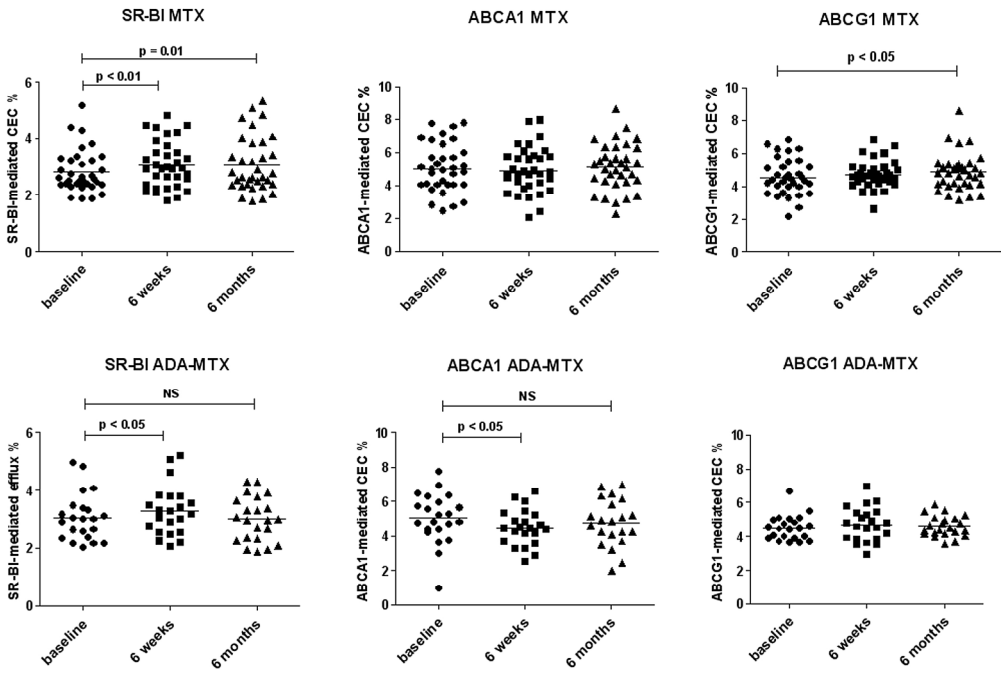


Figure 1
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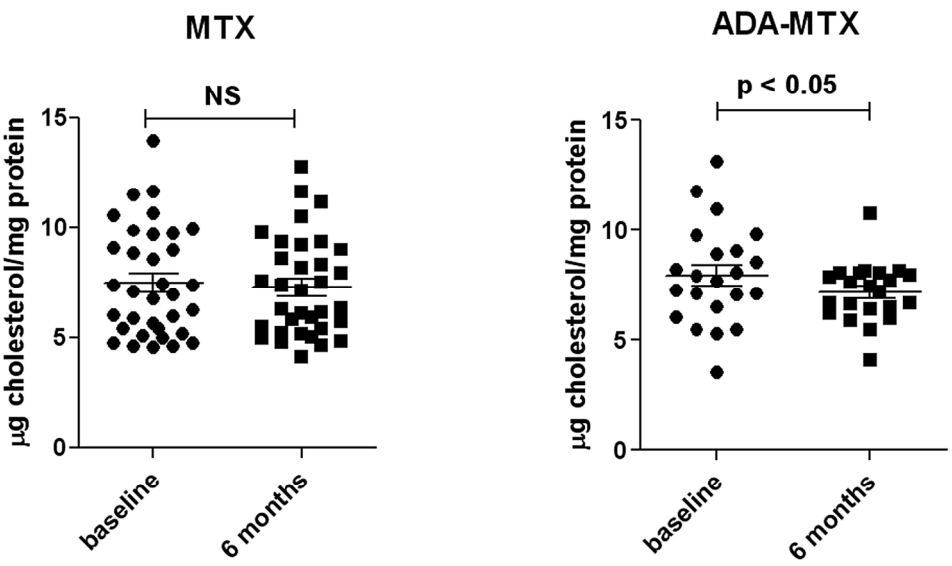


Figure 2
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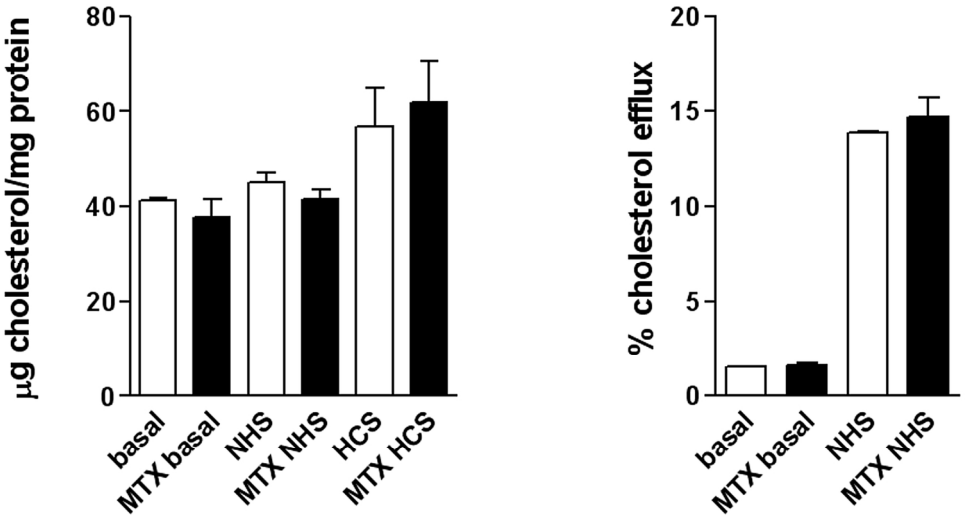


Figure 3
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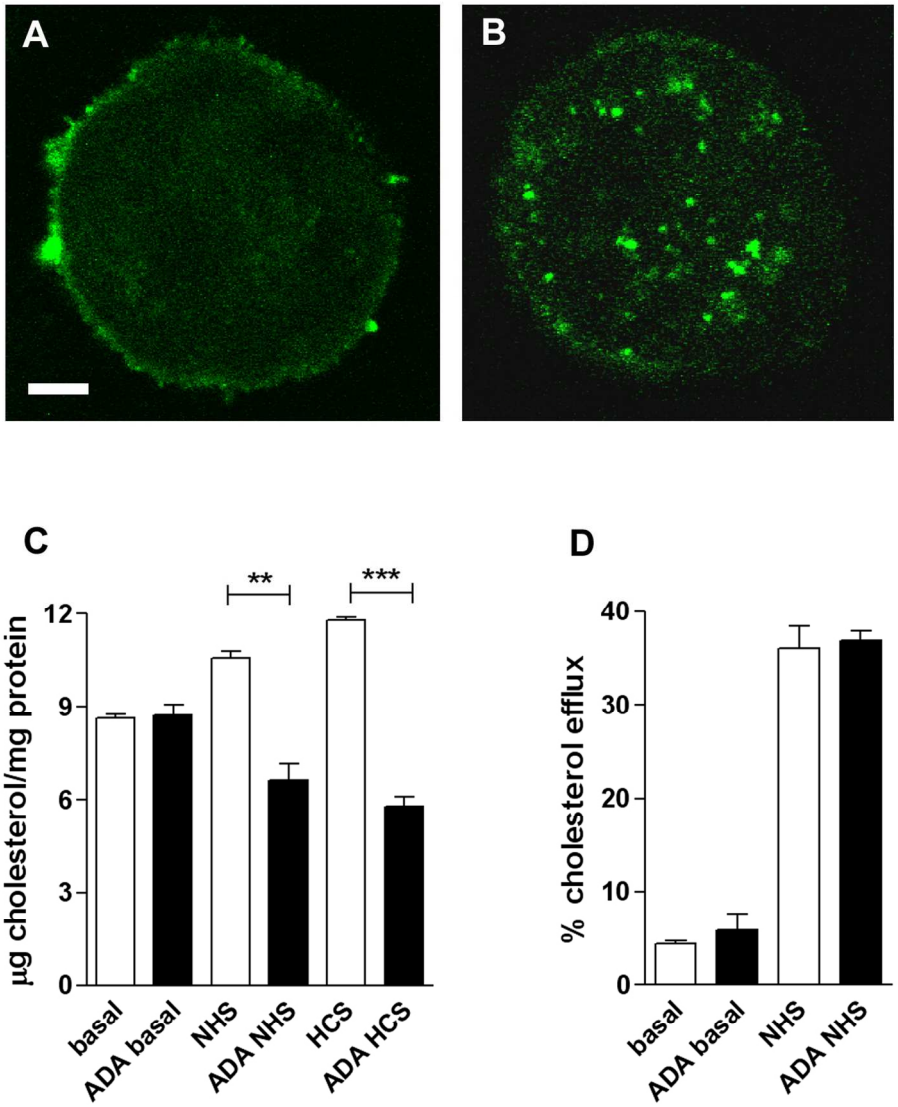


Figure 4
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**Table 1S.**

Clinical data of patients with RA at baseline.

| | MTX group, N (%) | ADA-MTX group, N (%) |
|---|---|---|
| Total | 34 | 22 |
| Female | 25 (73.5) | 16 (72.7) |
| Age (mean ± SE) | 56.3 ± 10.6 | 58 ± 8.2 |
| DD months (mean, range) | 3, 0.1-25 | 9, 0.5-30 |
| Previous CV events | 3 (8.8) | 4 (18.2) |
| Previous use of DMARDs | 13 (38.2) | 22 (100) |
| ESR (mean ± SE) | 26 ± 17.7 | 17 ± 11.6 |
| CRP (mean ± SE) | 15 ± 16.6 | 14 ± 18.6 |
| DAS28 (mean ± SE) | 5 ± 0.87 | 5 ± 1.24 |
| RF | 22 (64.7) | 16 (72.7) |
| ACPA | 17 (50.0) | 17 (77.3) |
| Hypertension | 7 (20.6) | 8 (36.4) |
| Diabetes | 0 (0) | 3 (13.6) |
| Smoking | 13 (38.2) | 4 (18.2) |
| MTX dose (mg/week) (mean ± SE) | 18 ± 3.0 | 18 ± 6.8 |
| Glucocorticoid | 6 (17.6) | 6 (27.3) |
| NSAIDs | 26 (76.5) | 15 (68.2) |
| Aspirin | 4 (11.8) | 1 (4.5) |
| Statin | 6 (17.6) | 4 (18.2) |

DD = disease duration; CV = cardiovascular (stroke, myocardial infarction, coronary artery bypass grafting); ESR = erythrocyte sedimentation rate; CRP = C reactive protein; DAS28= Disease activity score 28, calculated with ESR; RF = Rheumatoid factor, ACPA= anti-citrullinated protein antibodies; NSAIDs = non-steroidal anti-inflammatory drugs.

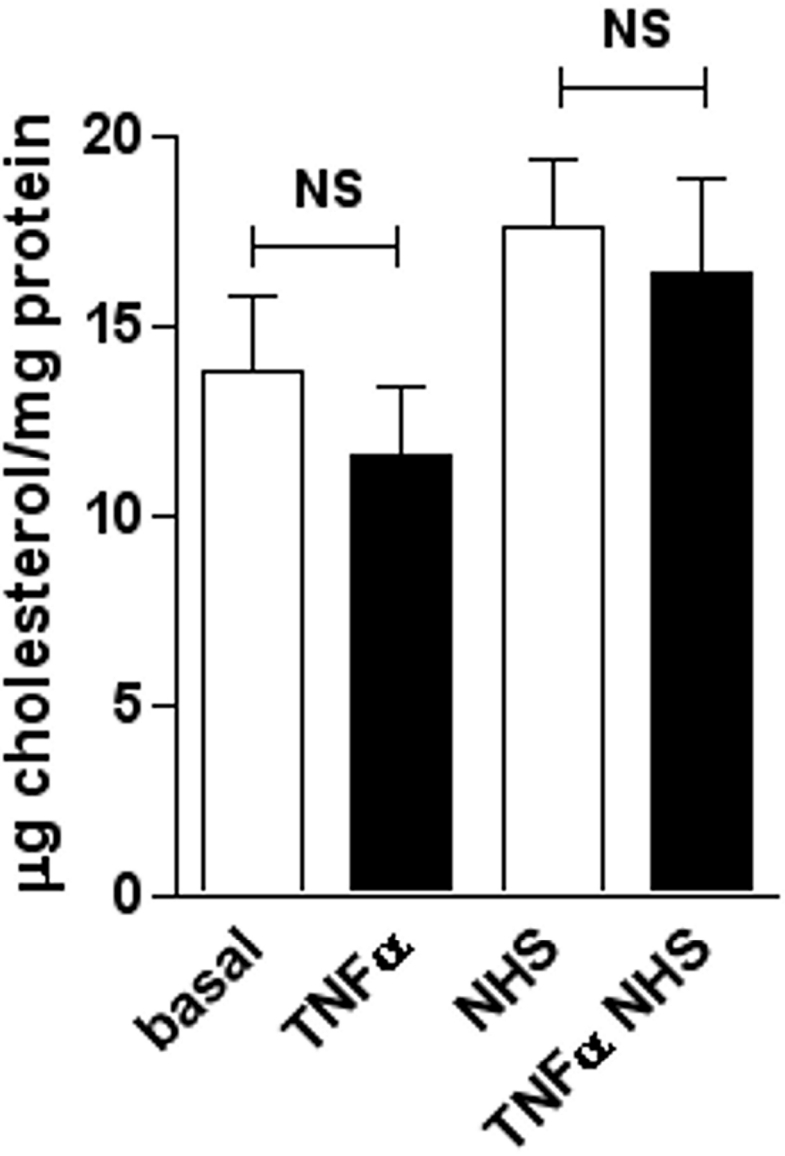


Figure 1S
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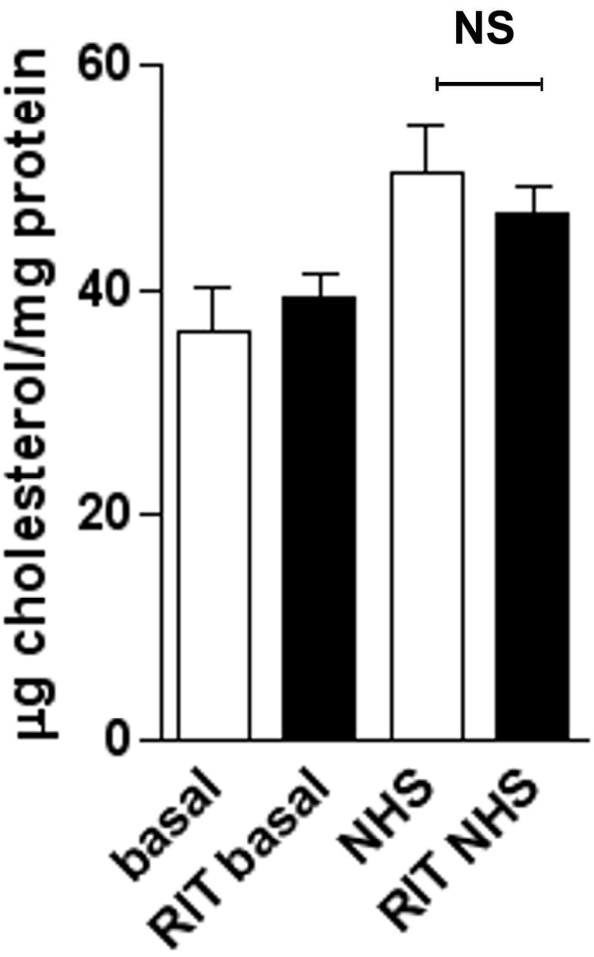


Figure 2S
74x109mm (600 x 600 DPI)