Low levels of PCSK9 are associated with remission in patients with Rheumatoid arthritis treated with anti- TNF-α: potential underlying mechanisms

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Abstract

Background

Proprotein convertase subtilisin kexin 9 (PCSK9) targets the LDL-receptor (LDLR) which raises LDL-levels. In addition, PCSK9 has proinflammatory immunological effects. Here we investigate the role of PCSK9 as to the inflammatory activity in patients with rheumatoid arthritis (RA).

Methods

PCSK9-levels were determined at baseline by ELISA in 160 patients with RA not previously treated with biologics. The patients started anti- TNF-α (adalimumab, infliximab, or etanercept) treatment and were followed up for one year. Disease activity was determined by DAS28.

Effects of PCSK9 on cytokine production from macrophages of healthy individuals and synoviocytes from RA patients and inhibition by anti-PCSK9 antibodies were studied in supernatants by ELISA.

Results

A significantly lower level of PCSK9 at baseline, p=0.035, was observed in patients who reached remission within one year, defined as DAS28<2.6, compared to those not in remission.

At 12 months of TNF-α antagonist treatment, the mean DAS28 was reduced but was significantly greater in patients with highest quartile PCSK9 (Q4) compared to those at lowest PCSK9 (Q1) in both crude (p=0.01) and adjusted analysis (p=0.004).

In vitro, PCSK9 induced TNF-alpha and IL-1beta in macrophages and monocyte chemoattractant protein-1 (MCP1) in synoviocytes. These effects were inhibited by anti-PCSK9 antibodies.

Conclusions

Low levels of PCSK9 at baseline is associated with being DAS28-responder to anti-TNF-α treatment in RA. An underlying cause could be that PCSK9 stimulates production of proinflammatory cytokines from macrophages and synoviocytes, effects inhibited by anti-PCSK9 antibodies. PCSK9 could thus play an immunological role in RA.

Key words

Proprotein convertase subtilisin kexin 9 (PCSK9), rheumatoid arthritis, disease activity, tumour necrosis factor (TNF), macrophages, synoviocytes

Introduction:

Rheumatoid arthritis (RA) affects 0.5 to 1% of total population, placing a substantial burden not only on the affected individuals but also on society.[1] Tumor necrosis factor α (TNF-α) antagonists are used as monotherapy as well as in combination with conventional antirheumatic drugs such as metotrexate.[2] These antagonists block the interaction of TNF-α with its receptors on cell surface, thereby lowering the systemic and local levels of pro-inflammatory cytokines, preventing infiltration of leukocytes and lymphocytes to the sites of inflammation, promoting inhibition of nuclear factor-KB, inducing apoptosis of TNF-α -producing cells, lowering the level of endothelial adhesion molecules and improving endothelial function. Still, about 30% of patients do not respond to treatment.[3]

Patients with RA have an increased risk of atherosclerosis complications causing cardiovascular disease (CVD) and also increased atherosclerosis progress and likely also plaque vulnerability, though there is a variation in reports.[4, 5] Biologics may prevent CVD in RA by ameliorating atherosclerosis complications.[6]

Proprotein convertase subtilisin kexin 9 (PCSK9) was identified as a novel factor influencing LDL-metabolism. Genetic variants leading to low PCSK9 activity through missense variants and were associated with low risk of CVD [7, 8] while other genetic variants led to high PCSK9 activity and high levels of LDL.[9] A mechanism was identified where PCSK9 targets the LDL-receptor (LDLR), and ensuing decreased activity of LDLR leads to increased LDL-levels and inhibition of PCSK9 is now used to treat CVD-patients.[10-13]

Both statins and inhibition of PCSK9 may have other effects than LDL-lowering. They both inhibit Oxidized LDL (OxLDL)-mediated immune activation through similar but not identical mechanisms.[14, 15] OxLDL, together with dead cells and activated immune competent cells, producing mainly proinflammatory cytokines are key elements of atherosclerotic plaques.[16]

Earlier we have reported that PCSK9-levels are raised among patients with high disease activity in systemic lupus erythematosus (SLE), and that OxLDL induced PCSK9 in dendritic cells (DC), effects which were significantly stronger in DCs from SLE patients than from controls.[17] OxLDL, which is increased in SLE, induced PCSK9, an effect which was higher among SLE patients. We suggested that PCSK9 could play an imunological role in SLE.[17] Also other studies demonstrate pro-inflammatory effects of PCSK9.[15, 18]

We here report that low PCSK9-levels at start of anti-TNF treatment in patients with RA are associated with being a responder to that therapy and elucidate potential underlying mechanisms. The implications are discussed.

Materials and methods:

Patients and healthy individuals

One hundred and sixty outpatients who satisfied American College of Rheumatology (ACR) criteria for Rheumatoid Arthritis (RA) and were not previously treated with biologics, were recruited for the study at the Rheumatology Department, Karolinska University Hospital Huddinge. The patients were administered anti- TNF-α (adalimumab, infliximab, or etanercept) for at least one year. The patients were followed and disease activity was assessed at treatment initiation time 0 and at 3, 6 and 12 months. Disease activity was assessed by the composite index Disease Activity Score calculated in 28 joints (DAS28; range 0–9.4, best to worse). This composite index includes number of swollen joints, number of tender joints, patient's global assessment of disease activity measured on a visual analogue and erythrocyte sedimentation rate (ESR). Remission was defined as DAS28 <2.6 according to the European League Against Rheumatism (EULAR) criteria.[19] A Swedish version of the Stanford Health Assessment Questionnaire (HAQ) [20] was used to assess functional disability. In addition, information on body mass index (BMI; kg/m2), hypertension, rheumatoid factor (RF) positivity, current smoking, history of diabetes mellitus, CVD (myocardial infarction, congestive heart failure, angina pectoris, and ischemic stroke) and current medications was collected.

The ethics committee at Karolinska Institute, Stockholm, Sweden has approved this study. The study was performed according to the Helsinki declaration. All participants provided written informed consent.

PCSK9-measures in serum

Sera were collected at baseline and preserved at -80°C until analysis was done. Enzyme linked immunosorbent assay (ELISA) was employed to determine the PCSK9 level from all sera at baseline. ELISA was performed by using commercial kit (R&D Systems, UK) following the manufacturer's protocol. PCSK9 levels were expressed as picogram per milliliter (pg/ml).

Cell culture

Macrophage culture

Buffy coats of healthy individuals were collected from Karolinska University Hospital, Sweden. Monocytes were isolated from the buffy coats and differentiated into macrophages with GM-CSF for 5 days and were stimulated with various concentration of endotoxin-free PCSK9 (Sigma Aldrich) in presence or absence of 5µg/ml of anti-PCSK9 antibodies (Amgen)

for 24 hours. The experiments were performed at least three times with cells (macrophages) of three individuals (n=3 in each experiment)

Synoviocytes

Human fibroblast-like synoviocytes from Rheumatoid arthritis patients were purchased from Lonza and cell passage 1 or 2 were cultured with DMEM media with 10% FBS and stimulated with various concentration of PCSK9 in presence or absence of anti-PCSK9 antibodies for 24 hours. Anti-PCSK9 antibodies were provided by Amgen

Experiments were performed three times and triplicates in each experiment.

Cytokines IL-1beta, TNF-α, and Monocyte chemoattractant protein-1 (MCP-1) were measured from cell cultured supernatants by ELISA duoset (R&D, Biotechne) according to manufacturer´s instruction. The experiments were performed with the cell passage number 1 and 2. All experiments were performed separately at least three times with at least triplicates in the separate experiments.

Anti-PCSK9 antibodies were provided by Amgen

Statistical analysis

The association of PCSK9-levels with disease activity score of 28 joints (DAS28) and functional ability (HAQ scores) of the TNF–α antagonist treated RA patients were assessed at baseline and after 3, 6 and 12 months. The data were analyzed by using generalized linear model. In order to obtain the association of PCSK9 at baseline with the outcomes of TNF–α antagonist treatment over time, we estimated the outcome (DAS28), at each follow up.

Patients were categorized into quartiles (Q1, Q2, Q3, and Q4) based on the distribution of PCSK9 at baseline. For each follow up (0 to 3, 0 to 6 and 0 to 12), variation in improvement of DAS28 within quartiles was calculated from crude models and models adjusted for potential confounding factors we chose to adjust for the same factors as in a larger previous study, sex, diabetes mellitus, hypertension, and CV history, which are known to be associated with PCSK9[21] Age was not associated with PCSK9 herein. The values of $p<0.05$ was considered statistically significant. These analyses were performed using SAS 9.4 release (SAS Institute, Cary, NC).

Data from cell culture experiments were presented in a bar diagram with standard deviation. Statistical analysis was performed by one way ANOVA.

Results

Patients' demographic and clinical characteristics

Patients' demographic and clinical characteristics are studied and summarized in Table 1. About three times higher number of patients (35.9%) in the first quartile (lowest level of PCSK9) achieved remission after one year compared to those with highest level of PCSK9 (12.8%) [risk ratio 0.36 (95% CI 0.15, 0.9)]. The odds of reaching remission in lowest PCSK9 patients increased significantly (74%) compared to those at highest PCSK9 category [odds ratio 0.26 (95% CI 0.08, 0.82). The characteristics of patients administered different anti-TNF-α drugs such as adalimumab, infliximab and etanercept were similar (data not shown). There was no association between PCSK9 levels and DAS28 at baseline (data not shown).

Distribution of PCSK9 among quartiles in the RA patients before TNF–α antagonist treatment:

Upon distribution of the patients into quartiles based on their baseline levels of PCSK9, the median values of PCSK9 in the first, second, third and fourth quartile were found 102640 Pg/ml (IQR: 29654), 141288 Pg/ml (IQR: 22124), 189960 Pg/ml (IQR: 19342) and 259928 Pg/ml (IQR: 65704) respectively.

Association between baseline PCSK9 level and DAS28 in RA patients.

After one year of treatment, DAS28 values were generally decreased, with 43 patients (27%) achieving remission target (DAS28<2.6). The PCSK9 levels of patients at baseline who achieved remission were compared with those not in remission after one year. A significantly lower level of PCSK9 level at baseline, p=0.035, was observed in patients who reached remission after one year compared to those not in remission (Figure 1)

In patients in the lowest PCSK9 category at baseline (first quartile, Q1) the mean DAS28 after 3 months was found to have 0.32, 0.46- and 0.45-units greater reductions than the higher PCSK9 categories; second, third and fourth quartiles (Q2, Q3, and Q4) respectively (Table 2. This was even greater after 6 months following the same clear trend of baseline PCSK9 dependent DAS28 change as it was after 3 months. At 12 months of TNF–α antagonist treatment, the mean DAS28 was found reduced in all PCSK9 categories but DAS reduction remained significantly greater in patients with highest PCSK9 (Q4) compared to those at lowest PCSK9 (Q1) in both crude (p=0.01) and adjusted analysis (p=0.004). Moreover, the mean DAS28 reduction was also found significantly higher in patients at Q3 compared to those at lowest PCSK9 category in adjusted analysis (p=0.03; Table 2).

After one year 35.9% patients at lowest PCSK9 category achieved remission, which was significantly higher compared to those at highest PCSK9 category where only 12.8% achieved remission [risk ratio 0.36 95% CI 0.15, 0.9)] (Table 2)

A statistically significant increase in the odds of reaching remission in patients at lowest PCSK9 category compared to those at highest PCSK9 category was observed by logistic regression after one-year treatment [odds ratio 0.26 (95% CI 0.08, 0.82 (Table 2)

Associations between PCSK9 at baseline and ESR, CRP and HAQ

Functional ability (HAQ) of patients in different categories were also found following the same trend except for those patients in Q3 category whom experienced a greater mean reduction in both CRP, HAQ than highest PCSK9 category but did not achieve statistical significance (Table 3).

PCSK9 induced pro-inflammatory effects and inhibition by anti-PCSK9 antibodies

PCSK9 induced TNF-α, and IL-1beta in macrophages in a dose dependent way (Figure 2a) and this was inhibited by anti-PCSK9 antibodies (Figure 2b). The experiments were performed three times with cells obtained from three individual donors and experiments on cells of all donors showed similar results. Experiments from cells of one donor is showed as a representative.

PCSK9 induced MCP1 in synoviocytes in a dose dependent way (Figure 3a), and this effect was inhibited by anti-PCSK9 antibodies (Figure 3b). The experiments on synoviocytes (passage 1and 2) performed three times and mean value of three experiments are presented. TNF- α could not be detected from synoviocytes,

Discussion

We here report that baseline levels of PCSK9 were negatively associated with disease activity as determined by DAS28 after 3 months, 6 months and 12 months among TNF–α antagonist-treated RA patients. The odds ratio after one year to be in remission among RA patients in the lowest quartile of PCSK9-levels at baseline was four times higher than for those in the highest quartile of PCSK9 levels,

Despite advances during recent years, the treatment of RA remains a challenge. Anti- TNF-α therapy is the most common biologic treatment for RA patients, but the rate of remission is not satisfactory and about 30% of patients are non-responders.[22]

Our findings could have clinical implications, both to improve prediction of effects of TNF-α-inhibition in RA and to improve prediction of outcome in order to optimize therapy, because one of the problems is the difficulty to identify non-responders before treatment,. Our findings may imply that RA patients with low baseline levels of circulating PCSK9 could respond to TNF–α antagonist treatment significantly better than those with higher baseline PCSK9.

It is also possible that RA-patients with high PCSK9 levels could be eligible for other types of therapy, or combinations with TNF-α-inhibition at an early stage, depending on whether other treatments, as established disease modifying anti-rheumatic drugs (DMARDs) or novel biological therapies are associated with baseline PCSK9-levels.

Another question is whether PCSK9 and its inhibition also could play a role in underlying mechanisms and in immune reactions and inflammation causing RA. We therefore investigated the effects of PCSK9 on activation of cell types which are implicated in RA-synovia and generally believed to play an important role in disease development.

In the synovial joint of RA patients, activated macrophages, neutrophils and lymphocytes are abundant and play an important role in the disease pathogenesis and most likely also activated synoviocytes play an essential role in local inflammation.[23, 24] To investigate potential underlying mechanism of anti- TNF-α treatment failure, we studied effects of PCSK9, in physiological concentrations in similar range as those present in the circulation, on macrophages and synoviocytes.

We determined that PCSK9 induces pro-inflammatory TNF-α and IL-1-beta in a concentration dependent manner from macrophages. The finding could thus shed light on the lack of response to anti- TNF-α therapy in RA-patients with high PCSK9-levels, since PCSK9 could counter the anti- TNF-α effects. We could not detect TNF-α from the synoviocytes.

MCP1 may play an important role in RA pathogenesis, including recruitment of macrophages. [25] Further, inhibition of MCP-1 ameliorated arthritis in rat models.[26] Also levels of MCP-1 in

sera of RA-patients is raised.[25] Still the role and induction of MCP-1 in RA is poorly understood. We here report that PCSK9 induced MCP-1 from synoviocytes, and it is thus possible that PCSK9 could contribute to MCP-1 induction at least in subgroups of RA patients. We speculate that PCSK9-induced synoviocytes recruit leucocytes including macrophages through MCP-1 to the site of inflammation and thus increasing the risk of failed anti- TNF-α therapy.

These effects – induction of TNF-α and IL-1 beta from macrophages and MCP-1 in synoviocytes, were inhibited by antibodies against PCSK9. It is thus possible that inhibition of PCSK9 could contribute to amelioration of chronic inflammation as in RA, especially in individuals with high levels of PCSK9. Clinical studies are needed to study this possibility.

These findings could also shed light on the increased risk of atherosclerosis and CVD which is described in RA, and also on the inflammation in atherosclerotic plaques.

A combination of traditional risk factors, as diabetes, hypertension and smoking and non-traditional risk factor as inflammation may account for the increased risk of CVD including stroke and myocardial infarction (MI). [26]

Atherosclerosis is characterized by the presence of activated immune competent cells, but also necrotic core of dead cells and OxLDL, mostly engulfed in foam cells.[27] OxLDL induces activation of monocytes and T cells [28,29], also from human atherosclerotic plaque-derived T cells and dendritic cells (DC).[30] OxLDL has been detected in foam cells in rheumatic synovia [31], and OxLDL in the circulation is raised in RA and also associated with CVD in RA.[32]

In previous studies, we demonstrated that OxLDL-induced pro-inflammatory cytokines IFN-δ and IL-17 in a complex immune reaction where heat shock protein 60/65 plays a role.[30] PCSK9 plays a key role in OxLDL-induced pro-inflammatory effect by dendritic cells (DCs).[15]. PCSK9-silencing inhibited OxLDL-induced activation of DCs and subsequently induction of regulatory T-cells (T-regs) and IL-10 cytokines [15].

T-reg cells could be of major importance in autoimmune diseases, such as RA, by suppressing several immune cells, including CD8+ and CD4+ T-cells, antigen presenting cells (APCs), natural killer (NK) cells and dendritic cells.[33]. Therefore, RA ameliorating T-reg cells and anti-inflammatory cytokine IL-10 demolishing effects of PCSK9 could contribute to modification of anti TNF–α treatments in RA patients.

There are limitations to this study. We were not able to differentiate between three different TNF-α-antagonists due to the size of the study population and it would be interesting to

investigate also other novel treatment modalities in RA. Further, the numbers of patients in remission per studied PCSK9 quartile are relatively low. The in vitro experiments are hypothesis-generating, but studies with PCSK9-inhibition in RA are necessary to establish PCSK9 as an underlying agent in this disease (and other inflammatory conditions). We only followed up patients for one year. Longer follow up time is needed to establish if the associations remain between disease activity and outcome, as here in DAS28, but also for other parameters related to the disease including HAQ.

Conclusions

Taken together, the results indicate that PCSK9 could play a role in predicting outcome in RA, and also that PCSK9-inhibition could be of interest therapeutically in RA, a possibility deserving further studies.

List of abbreviations

PCSK9: (Proprotein convertase subtilisin kexin 9)

LDL: low density lipoprotein

Ox: oxidized

TNF: tumor necrosis factor

RA: rheumatoid arthritis

CRP: C-reactive protein

MCP-1: monocyte chemoattractant protein-1

DAS 28: disease activity score 28

HAQ: health assessment questionnaire

Declarations

Ethics approval and consent to participate

The ethics committee at Karolinska Institute, Stockholm, Sweden has approved this study. The study was performed according to the Helsinki declaration. All participants provided written informed consent.

Consent for publication

Not applicable since no individual data is presented

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

There is no conflict of interest for any of the authors. JF has research grant from Amgen, as indicated in the manuscript, but this is investigator-initiated and Amgen has no influence on the research or presentation.

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Authors' contributions

JF conceived the project and wrote the manuscript, SA and MR performed experimentes and co-wrote the manuscript, SA and IH revised the manuscript, contributed to clinical aspects and conclusions and conceived of and created the cohort.

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Tables and figures

Table 1. Clinical characteristics and baseline demographic of 160 RA patients

Age, yrs	56.2 ± 12.4
Female, n (%)	117 (72.2)
Duration of RA, yrs	7 (4-14)
RF-positivity, n (%)	132 (81.5)
Current smoking, n (%)	39 (24.1)
Hypertension, n (%)	90 (61.2)
Diabetes mellitus, n (%)	9 (5.6)
CVD comorbidity, n (%)	46 (28.4)
Statin use, n (%)	6 (3.7)
BMI, kg/m^2	25.2 ± 4.6
DAS28	5.7 ± 1.0
HAQ-score	1.3 ± 0.6
MTX use, n (%)	126 (77.8)
GC use, n (%)	54 (33.3)
GC, mg/day	5 (5 to 7.5)
NSAID use, n (%)	112 (69.1)

Values are mean values with sd or median values with IQR as indicated

RF: rheumatoid factor; CVD: cardiovascular disease; BMI: body mass index; DAS28: disease activity score of 28 joints; HAQ: health assessment questionnaire; GC: glucocorticoid; NSAID: non-steroidal anti-inflammatory drug; yrs: years; MTX: methotrexate.

Table 2. DAS28 values and number of patients who reached remission (DAS28<2.6) in the different PCSK9 quartiles.

Period (Month)	Q1 (N=40)		Q2 (N=40)		Q3 (N=40)		Q4 (N=40)	
	DAS28 (mean)	Remission (patients)	DAS28 (mean)	Remission (patients)	DAS28 (mean)	Remission (patients)	DAS28 (mean)	Remission (patients)
3	3.69	**8**	4.04	**3**	4.02	**3**	4.00	**5**
6	3.68	**8**	3.95	**5**	4.01	**5**	3.86	**3**
12	3.30	**14**	3.89	**9**	3.79	**10**	4.10	**5**

DAS28: disease activity score of 28 joints; Q1: first quartile; Q2: second quartile; Q3: third quartile; Q4: fourth quartile; N: number of patients.

Table 3. Crude and adjusted differences in mean reductions between quartiles at all follow up during TNF–α antagonist treatment for one year in RA patients.

Model	Period (Months)	Difference in mean decrease. Q1 – Q2	P value	Difference in mean decrease. Q1 – Q3	P value	Difference in mean decrease. Q1 – Q4	P value
DAS28							
Crude	0 to 3	0.3	0.27	0.43	0.13	0.37	0.18
	0 to 6	0.29	0.34	0.49	0.1	0.31	0.32
	0 to 12	0.56	0.1	0.67	0.06	0.91	**0.01**
Adjusted	0 to 3	0.32	0.23	0.46	0.11	0.45	0.12
	0 to 6	0.37	0.22	0.53	0.08	0.48	0.13
	0 to 12	0.65	0.06	0.76	**0.03**	1.06	**0.004**
CRP							
Crude	0 to 3	6.63	0.39	6.73	0.39	13.61	0.08
	0 to 6	3.11	0.63	2.82	0.67	5.66	0.39
	0 to 12	7.67	0.39	10.68	0.23	9.26	0.31
Adjusted	0 to 3	6.07	0.44	5.16	0.52	13.79	0.09
	0 to 6	3.67	0.58	3.04	0.65	6.98	0.31
	0 to 12	8.56	0.32	11.64	0.19	10.32	0.25
ESR							
Crude	0 to 3	0.14	0.96	1.9	0.6	5.89	0.11
	0 to 6	-1.87	0.64	1.07	0.79	0.68	0.86
	0 to 12	0.95	0.84	5.81	0.22	7.28	0.13
Adjusted	0 to 3	0.17	0.96	2.1	0.58	5.39	0.15
	0 to 6	-1.77	0.66	0.78	0.85	1.01	0.81
	0 to 12	1.43	0.76	6.87	0.16	6.98	0.15
HAQ							
Crude	0 to 3	-0.03	0.98	2.84	0.16	-0.06	0.97
	0 to 6	0.03	0.76	0.08	0.4	-0.003	0.97
	0 to 12	0.07	0.51	0.99	0.39	0.08	0.47
Adjusted	0 to 3	-0.03	0.98	2.81	0.18	0.05	0.98
	0 to 6	0.06	0.55	0.12	0.26	0.03	0.75
	0 to 12	0.1	0.36	0.13	0.24	0.14	0.25

DAS28: disease activity score of 28 joints; HAQ: health assessment questionnaire; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; Q1: first quartile; Q2: second quartile; Q3: third quartile; Q4: fourth quartile. Results are indicated as mean and the number of patients is as indicated in table 1. P-values are calculated from the difference in disease outcomes at different quartiles compared to Q1. Adjustment was done for sex, diabetes mellitus, hypertension and CV history

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Figure 1.

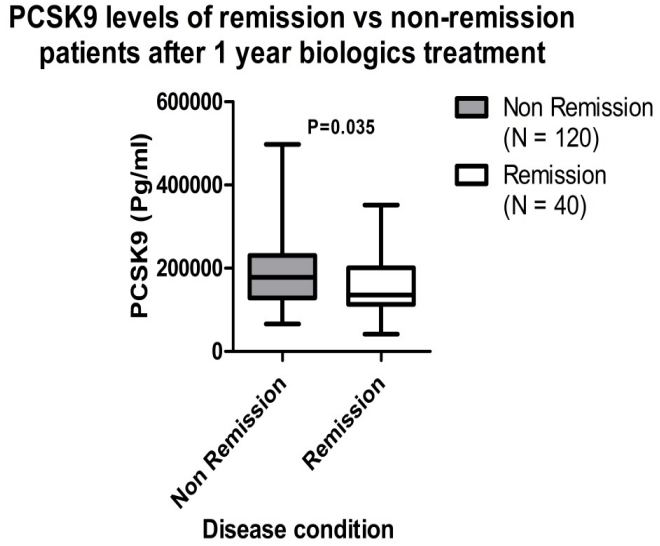

Figure 1. PCSK9-levels at baseline among TNF– α antagonist treated patients with remission (DAS<2.6) vs non-remission (DAS≥2.6) after 1 year. Results are presented as whiskers: min to max. Student´s t-test was performed for statistical analysis.

Figure 2a

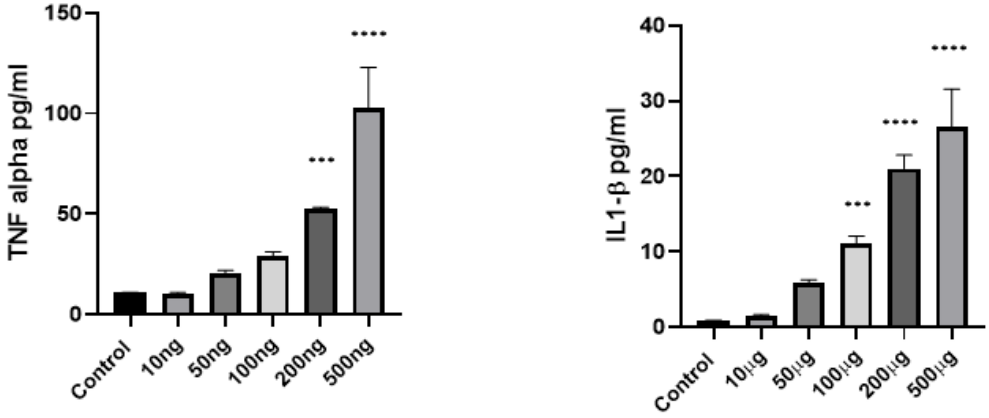

Figure 2b

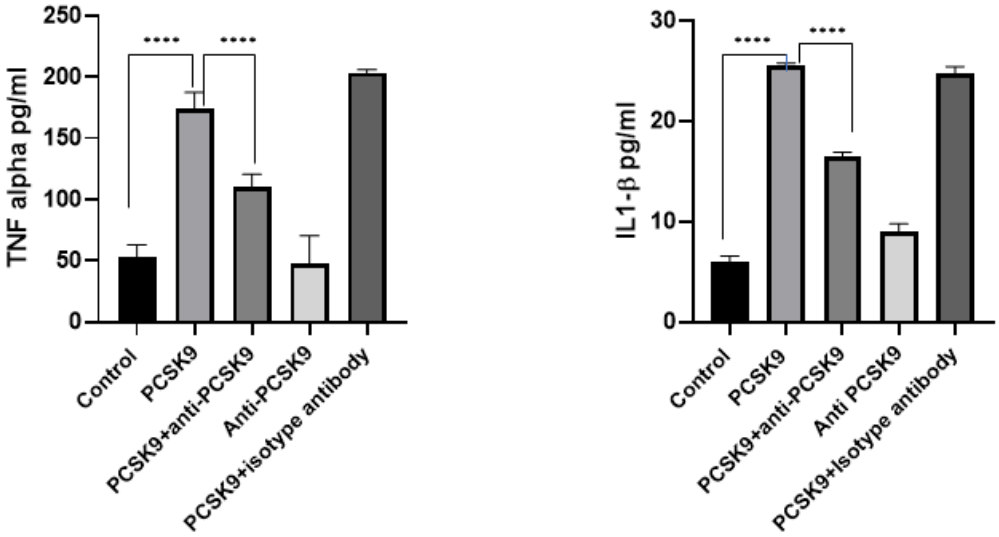

Figure 2: Macrophages were stimulated with PCSK9 in presence or absence of anti-PCSK9 antibodies. (a) Macrophages induced TNF-α and IL-1beta (n=3). (b)The level of TNF-α and IL-1beta was suppressed by anti-PCSK9 antibodies (n=3).

Figure 3a

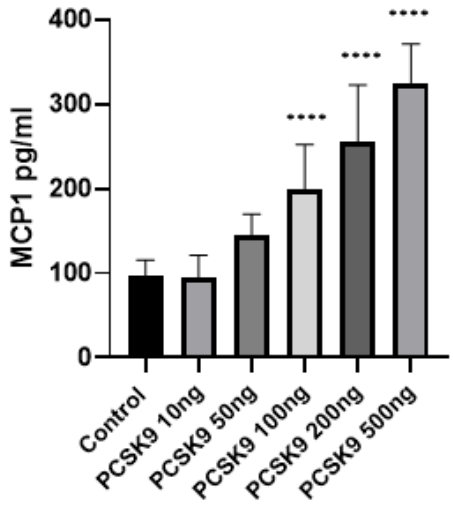

Figure 3b

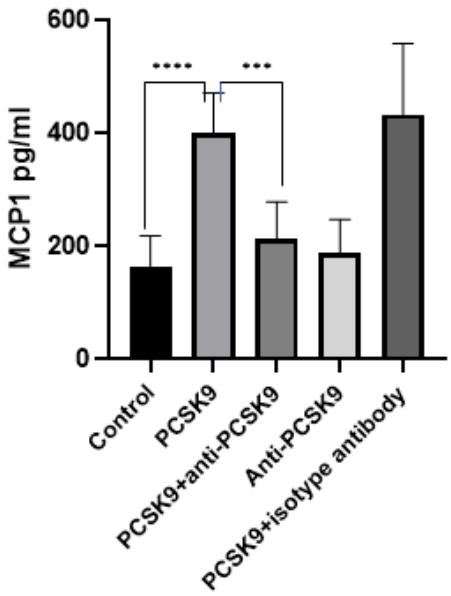

Figure 3. Synoviocytes were cultured with or without PCSK9 in the presence of anti-PCSK9 antibodies. (a) PCSK9 induced level of MCP1 in the synoviocytes (n=9), and (b) the level was inhibited by anti-PCSK9 antibodies (n=9).