Atherosclerosis 334 (2021) 93–100

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Colchicine reduces extracellular vesicle NLRP3 inflammasome protein levels in chronic coronary disease: A LoDoCo2 biomarker substudy

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ARTICLE INFO

Keywords:
Coronary disease
Colchicine
NLRP3 inflammasome
Extracellular vesicles

ABSTRACT

Background and aims: Colchicine reduces the risk of cardiovascular events in patients with coronary disease. Colchicine has broad anti-inflammatory effects and part of the atheroprotective effects have been suggested to be the result of NLRP3 inflammasome inhibition. We studied the effect of colchicine on extracellular vesicle (EV) NLRP3 protein levels and inflammatory markers, high sensitivity-CRP (hs-CRP) and interleukin (IL)-6, in patients with chronic coronary disease.

Methods: In vitro, the NLRP3 inflammasome was stimulated in PMA-differentiated- and undifferentiated THP-1 cells. *In vivo,* measurements were performed in serum obtained from 278 participants of the LoDoCo2 trial, one year after randomization to colchicine 0.5 mg once daily or placebo. EVs were isolated using precipitation. NLRP3 protein presence in EVs was confirmed using iodixanol density gradient centrifugation. Levels of NLRP3 protein, hs-CRP and IL-6 were measured using ELISA.

Results: In vitro, NLRP3 inflammasome stimulation showed an increase of EV NLRP3 protein levels.

EV NLRP3 protein levels were lower in patients treated with colchicine (median 1.38 ng/mL), compared to placebo (median 1.58 ng/mL) ($p = 0.025$). No difference was observed in serum NLRP3 protein levels. Serum hs-CRP levels were lower in patients treated with colchicine (median 0.80 mg/L) compared to placebo (median 1.34 mg/L) ($p < 0.005$). IL-6 levels were lower in patients treated with colchicine (median 2.07 ng/L) compared to placebo (median 2.59 ng/L), although this was not statistically significant ($p = 0.076$).

Conclusions: Colchicine leads to a reduction of EV NLRP3 protein levels. This indicates that inhibitory effects on the NLRP3 inflammasome might contribute to the atheroprotective effects of colchicine in coronary disease.

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https://doi.org/10.1016/j.atherosclerosis.2021.08.005
Received 4 February 2021; Received in revised form 27 June 2021; Accepted 4 August 2021
Available online 11 August 2021

1. Introduction

Inflammation is one of the major processes underlying the progression of atherosclerosis leading to atherothrombotic complications such as myocardial infarction and stroke [1,2]. An effective anti-inflammatory treatment for atherosclerosis, reducing cardiovascular events independent of lipid lowering, proved elusive for decades.

The Canakinumab Antiinflammatory Thrombosis Outcome Study (CANTOS) demonstrated that targeted anti-inflammatory therapy with selective inhibition of interleukin (IL)-1β reduced the risk of major cardiovascular events in patients with recent myocardial infarction [3]. Subsequently, the Colchicine Cardiovascular Outcomes Trial (COLCOT) showed that 0.5 mg of colchicine, an anti-inflammatory drug used for gout, pericarditis and familial Mediterranean fever, administered once daily reduced the risk of cardiovascular events in patients with recent myocardial infarction [4]. Recently, the second Low-Dose Colchicine (LoDoCo2) trial demonstrated that a similar dose of colchicine reduced the risk of cardiovascular events in patients with chronic coronary disease [5].

Colchicine is a drug with broad anti-inflammatory effects and, although it has been used for centuries, its exact mechanisms of action remain unclear. Among others, colchicine acts through the inhibition of tubulin polymerization, inhibition of adhesion molecules and cytokines, and the inhibition of the nucleotide-binding oligomerization domain-, leucine-rich repeat-, and pyrin domain-containing protein 3 (NLRP3) inflammasome [6,7]. NLRP3 is an intracellular innate receptor that, after activation, forms the intracellular inflammasome multiprotein complex that results in caspase 1-dependent cleavage and subsequent release of the mature pro-inflammatory cytokines IL-1β and IL-18 [8–10]. Both IL-1β and IL-18 are known to drive atherosclerotic plaque progression and increase the risk for instability [9,11,12]. IL-1β levels are generally low and cleavage of its precursor pro-IL-1β is driven by several other pathways aside from the NLRP3 inflammasome [13–15]. IL-1β thus proves to be a difficult indicator for NLRP3 inflammasome activity.

NLRP3 inflammasome activation also results in the secretion of its intracellular components, including NLRP3 protein, in extracellular vesicles (EVs) [16,17]. EVs are small bilayer membrane vesicles including microvesicles, microparticles and exosomes, ranging from 50 to 1000 nm in diameter, that are released during cell activation or apoptosis [18,19]. EVs contain bioactive content (proteins, lipids, nucleic acids) from their parent cell and are acknowledged messengers for intercellular communication [18].

We hypothesized that NLRP3 inflammasome stimulation leads to increased NLRP3 protein levels in EVs and that inhibition with colchicine attenuates NLRP3 inflammasome stimulation and reduces circulating EV NLRP3 protein levels and the downstream inflammatory markers high sensitivity C-reactive protein (hs-CRP) and IL-6 in patients with chronic coronary disease.

2. Materials and methods

2.1. Cell culture maintenance, preparation and stimulation

Cells from the human monocytic cell line THP-1 were obtained from the American Type Culture Collection (ATCC, United States of America) and maintained in Roswell Park Memorial Institute 1640 medium, GlutaMax supplement (Gibco), supplemented with 10% heat-inactivated Fetal Bovine Serum (1 h at 56 °C, Biowest) and 1% pen/strep (Gibco) solution). The cell culture medium was replaced every two days and cultures were maintained at 37 °C in a 5% CO2 humidified incubator.

2.2. NLRP3 inflammasome stimulation in vitro

For NLRP3 inflammasome stimulation, THP-1 cells (2 x 10^6 cells/well) were plated in six-well culture plates in serum-free medium. For differentiation of the THP-1 monocytes into macrophages THP-1 cells were incubated with 100 ng/mL of phorbol 12-myristate 13-acetate (PMA) for two days. THP-1 cells (non-adherent monocytes or attached macrophages) were incubated with 100 ng/mL of lipopolysaccharide (LPS) (Sigma Aldrich) for 3 h. Hereafter, 5 mM of adenosine triphosphate (ATP) (Sigma Aldrich) was added and cells were incubated for another 13 h for non-adherent monocytes and 3 h for attached macrophages. For the cells treated with colchicine, cells were plated (2 x 10^6 cells/well) in serum-free medium with either 0.2 μM, 1,0 μM or 10 μM colchicine 30 min prior to NLRP3 inflammasome stimulation. Cell viability was examined using trypan blue (Sigma Aldrich) in a trypan blue exclusion assay. The cell culture supernatant was collected and stored at −80 °C.

2.3. Platelet activation

Healthy controls were recruited through the Minidonor service, an institutional Review Board approved biobank at the University Medical Center Utrecht (biobank protocol number 18-774). All donors gave written informed consent in accordance with the declaration of Helsinki. Whole blood was collected through venepuncture in BD vacutainer tubes containing 1/10th volume trisodium citrate (104 mM) and subsequently centrifuged at 160*g* for 15 min with the break set at 1 to obtain platelet rich plasma (PRP). PRP was transferred to fresh tubes and subsequently incubated with either 10 μM colchicine or an equal volume of placebo for 30 min at 37 °C. Next, PRP samples were transferred to an aggregometer (Chrono-log, Havertown, PA, USA) and stimulated with 25 μM Thrombin Receptor Activating Peptide-6 (TRAP-6; SFLLRN; Bachem, Bubendorf, Switzerland) to allow full aggregation. Platelet poor plasma (obtained by centrifugation at 2000*g* for 10 min) was used as blank. Platelet aggregation was monitored for 30 min to allow release of EVs. Finally, supernatant was collected, centrifuged twice at 2000*g* for 15 min to remove platelet aggregates and plasma was stored at −80 °C for vesicle isolation.

2.4. Study design and population

We conducted a pre-defined biomarker substudy of the second Low-Dose Colchicine for the secondary prevention of cardiovascular events (LoDoCo2) trial (ACTRN12614000093684). The methods and main results have been published before [5,20]. In short, patients were eligible for participation if they were between 35 and 82 years old, had evidence of coronary disease and were clinically stable. All patients entered an open label run-in phase and, if they perceived no side effects of the drug and were willing to continue, were randomized to colchicine 0.5 mg once daily or placebo, on top of regular care. Randomization was performed in a double-blind manner with the use of a computerized algorithm. Main exclusion criteria were an impaired renal function, defined as an estimated glomerular filtration rate (eGFR<50 ml/min/1,73 m^2) or a serum creatinine >150 μmol/L, the need to take colchicine for any other indication, or concomitant drug use of strong Cytochrome P450 3A4 (CYP3A4) inhibiting drugs (i.e. verapamil, azithromycin, clarithromycin) [20].

Participants of this substudy were included in three random Dutch hospitals with laboratory facilities (Northwest Clinics, Alkmaar; Meander Medical Center, Amersfoort and Treant Hospital, Emmen, The

Netherlands). The study protocol was approved by a medical ethics committee (MEC-U, Nieuwegein, The Netherlands). All participants provided written informed consent prior to enrollment in the study. The study was conducted according to the Declaration of Helsinki. Data supporting the findings of this study are available from the corresponding author upon reasonable request.

2.5. Laboratory assessment

Blood samples were drawn in participants one year after randomized treatment with colchicine or placebo. All samples were centrifuged at 1500*g* at 4 °C for 15 min, and serum was stored at −80 °C.

2.5.1. Isolation of extracellular subfractions

Serum EV or cell culture medium EV subfractions were isolated using a standardized protocol [21,22].

For the total extracellular vesicle (TEX) subfraction, 25 μL of serum was diluted in 95 μL PBS and 5 μL Nano-mag®-D PEG-OH (1:25) (Micromod). The serum EV were then precipitated using Xtractt buffer (1:4) (Cavadis BV).

2.5.2. Characterization of extracellular vesicles

Iodixanol density gradient centrifugation of the subfraction was used to determine in which density fraction NLRP3 protein was present in the TEX fraction. Characterization of the density fractions of TEX isolation was described in detail before [21,22]. In these previous studies, we show that with the precipitation method we used, after density gradient centrifugation there was CD9 positivity (Western blot) in the 1,05-1,09 density. In these densities, visible bilayer vesicles (electron microscopy) were present, and NLRP3 protein was measured. This reveals the presence of EVs with NLRP3. In the current study, all TEX density gradient fractions were characterized by CD9 Western blot analysis with CD9 as EV protein marker. To get easy access to the overall data on characterization after density gradient, an EV-track ID was created: EV200044 [22].

2.5.3. Western Blot

Western blot analysis was performed on a 4-12% gradient Bis/Tris gel (NuPage, Invitrogen). After blotting, the blot was incubated with the following antibodies:

1. Primary antibody: CD9 antibody (purified Mouse anti-CD9 (MAB25 292) (R&D systems).
2. Secondary antibody: GOAT anti-mouse-HRP 1/1000) (P0447) (DAKO)

Visualization was done with a Super Signal enhanced luminol-based chemiluminescent kit (Thermoscientific) and image capture and analysis was done on a Biorad Chemidoc MP.

2.5.4. NLRP3 and interleukin-1β measurements

NLRP3 was quantified using a Human NLRP3 Enzyme-Linked Immunosorbent Assay (ELISA) kit (cat. No. EH4202, Fine Test, Fine Biological Technology, Wuhan, China) according to the manufacturer's instruction. The kit had a sensitivity of 0.47 ng/mL and a range of 0.78–50.0 ng/mL.

NLRP3 was measured in EVs and in a random selection (2/3) of patients in serum without EV isolation. *In vitro*, IL-1β was measured using a research IL-1β kit (Human IL-1 beta/IL-1F2 Quantikine HS ELISA Kit, R&D Systems, Minneapolis, MN, USA). This assay had a sensitivity of 0.063 pg/mL and an assay range from 0.1 to 8.0 pg/mL.

2.5.5. High sensitivity C-reactive protein and interleukin-6 measurements

Hs-CRP was measured using a research ELISA kit (Hycult Biotech #HK369, Uden, the Netherlands). The lower detection limit of this assay was 0.4 ng/L and the inter- and intra-assay coefficients of variation were <6.9% and <6.3%, respectively. IL-6 levels were measured by highly sensitive human IL-6 immunoassay (R&D Systems #D6050, Minneapolis, MN, USA). This assay had a sensitivity of 0.7 pg/mL and the intra-assay and inter-assay coefficients of variation ranged from 4.2% to 6.4%. This assay had a sensitivity of 0.7 pg/mL.

2.6. Statistical analysis

Continuous data are displayed using median values, and 25th and 75th percentile when non-normally distributed. Differences in continuous data were tested using unpaired t-tests when normally distributed or Mann-Whitney U tests when non-normally distributed. A Hodges–Lehmann estimator was used to provide a confidence interval (CI) for the estimated differences between non-parametric distributions. The linear relationship of two continuous parameters was assessed using the Spearman's rank correlation coefficient after data were log transformed. All statistical analyses were performed using IBM SPSS Version 25.0 (IBM Corp, Armonk, NY, USA).

3. Results

3.1. In vitro effects of NLRP3 inflammasome stimulation on EV NLRP3 protein levels

In vitro stimulation of the NLRP3 inflammasome in THP-1 cells showed higher EV NLRP3 protein levels (10.4 ng/ml ± 1.3 ng/ml) compared to unstimulated controls (6.6 ng/ml ± 0.5 ng/ml) (difference 3.8 ng/ml, 95% CI 0.4 to 7.0 ng/ml; p=0.03) (Fig. 1A). IL-1β levels in medium were higher in stimulated THP-1 cells compared to unstimulated control cells (Supplemental Fig. 1). *In vitro* stimulation of PMA-differentiated THP-1 cells showed higher EV NLRP3 protein levels (1.2 ng/ml ± 0.2 ng/ml) compared to unstimulated controls (0.36 ng/ml ± 0.02 ng/ml) (difference 0.84 ng/ml, 95% CI 0.27 to 0.70 ng/ml; $p < 0.0001$) (Fig. 1B). Cell viability was >90% for all conditions, without significant differences between groups. Colchicine treatment in different dosages in combination with NLRP3 inflammasome stimulation significantly affected cell viability (<30%). Platelet stimulation did not result in EV NLRP3 protein release (data not shown).

3.2. Laboratory assessment in LoDoCo2 participants

A total of 338 patients provided written informed consent and started the open-label run in phase. Forty-five patients (13.0%) were not randomized, most often due to perceived side effects (7.0%). All 293 patients that were randomized received at least one dose of the trial medication. With the exception of one participant, who died due to a non-cardiovascular cause, all patients were available for follow up at one year. In 7 patients in the colchicine group and in 7 patients in the placebo group, no blood sample could be obtained due to logistical reasons or on patient request. A total of 278 samples (n = 138 in the colchicine group and n = 140 in the placebo group) were available for analyses. A detailed flowchart is displayed in Fig. 2. The baseline characteristics of the patients were well balanced between groups. There were more women in the colchicine group (19.3%) compared to the placebo group (14.2%). Patients were well treated according to contemporary secondary prevention strategies (Table 1).

3.3. Extracellular vesicle NLRP3 protein level

Iodixanol density gradient centrifugation showed highest EV NLRP3 protein levels in the 3rd to 6th subfraction of this density gradient with the density of 1.03–1.11 g/ml. Subfractions with this density also contained the highest CD9 levels (Fig. 3), and thereby indicate that NLRP3 is expressed in EVs (Table 2).

EV NLRP3 protein levels were significantly lower in patients treated with colchicine (median 1.38 ng/mL) compared to placebo (median 1.58 ng/mL) (difference -0.20 ng/mL, 95% CI -0.37 to -0.03 ng/ml; $p = 0.025$) (Fig. 4). NLRP3 protein level in serum without EV isolation did not differ between treatment groups (Supplemental Fig. 2).

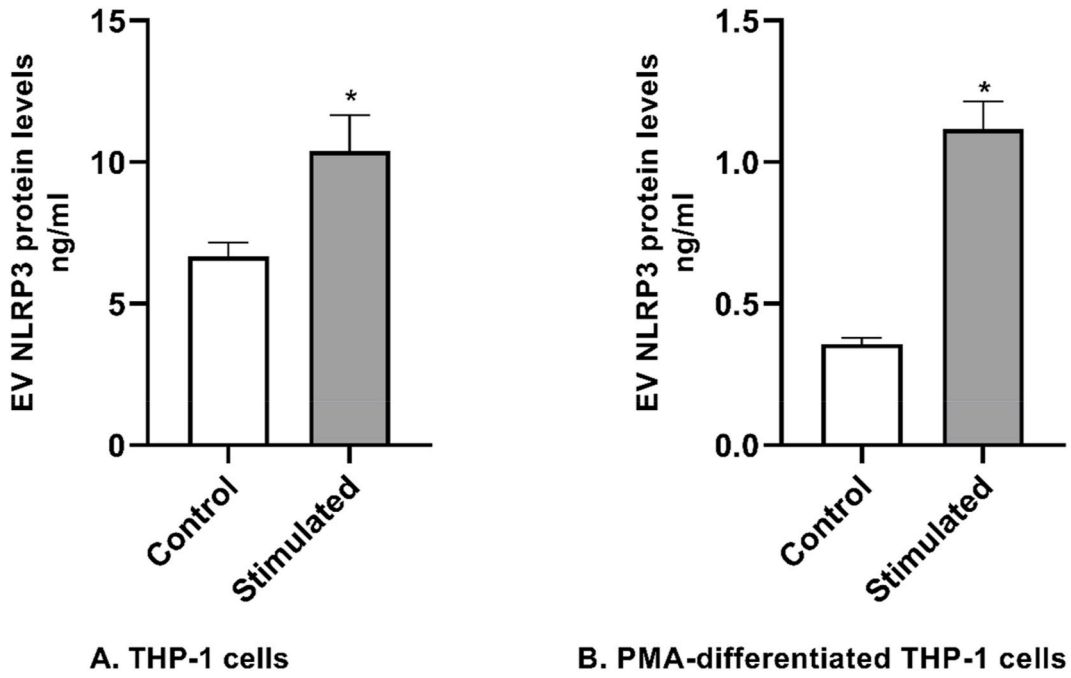

Fig. 1. *In vitro* effects of NLRP3 inflammasome stimulation and colchicine treatment on EV NLRP3 protein levels.
(A) THP-1 cells were stimulated with LPS and ATP (~16 h). Stimulation led to a significant increase in EV NLRP3 protein release compared to unstimulated controls.
(B) THP-1 cells were differentiated to macrophages using PMA. PMA-differentiated THP-1 cells were stimulated with LPS and ATP (6h). Stimulation led to a significant increase in EV NLRP3 protein release.
*p values < 0.05.
ATP, adenosine triphosphate; EV, extracellular vesicle; LPS, lipopolysaccharide; NLRP3, nucleotide-binding oligomerization domain-, leucine-rich repeat-, and pyrin domain-containing protein 3; PMA, phorbol 12-myristate 13-acetate.

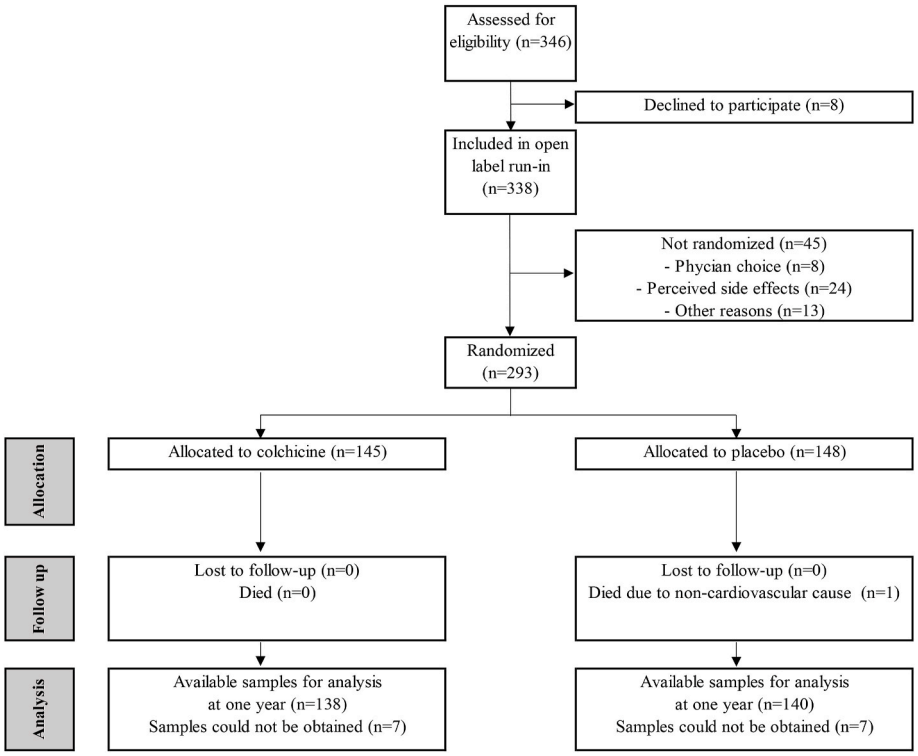

Fig. 2. Enrollment, randomization, and follow-up.
The figure shows the selection of subjects and reasons for exclusion.

Table 1
Patient characteristics.

Characteristic	Colchicine (N=145)		Placebo (N=148)	
Age – yr	66.1	±8.6	65.1	±9.3
Female sex – no. (%)	28	(19.3%)	21	(14.2%)
BMI – kg/m2	27.5	±4.1	27.7	±3.6
Current smoker – no. (%)[a]	16	(11.0%)	13	(8.8%)
Hypertension – no. (%)	80	(55.2%)	78	(52.7%)
Diabetes – no. (%)	31	(21.4%)	27	(18.2%)
Insulin dependent – no. (%)	9	(6.2%)	8	(5.4%)
Renal function[b]				
Stage 1, 2 – no. (%)	138	(95.2%)	139	(93.9%)
Stage 3a – no. (%)	7	(4.8%)	9	(6.1%)
History of acute coronary syndrome – no. (%)	119	(82.1%)	123	(83.1%)
Time since last acute coronary syndrome – yr	3.6	±5.2	4,9	±6.7
Coronary revascularization – no. (%)	138	(95.9%)	137	(92.6%)
History of atrial fibrillation – no. (%)	21	(14.5%)	20	(13.5%)
Medication use				
Single antiplatelet use – no. (%)	63	(43.4%)	60	(40.5%)
Dual antiplatelet use – no. (%)	70	(48.3%)	70	(47.3%)
Anticoagulant – no. (%)	21	(14.5%)	26	(17.6%)
Statin – no. (%)	130	(89.7%)	130	(89.7%)
Renin angiotensin inhibitor – no. (%)	107	(73.8%)	100	(67.6%)
Beta-blocker – no. (%)	101	(69.7%)	95	(64.2%)
Calcium-channel blocker – no. (%)	29	(20.0%)	36	(24.3%)

Plus-minus values are means and standard deviation.
BMI, body mass index; kg, kilogram; no, number SD, standard deviation.
[a] For 2 participants, information on smoking was missing.
[b] Stage 1 refers to an estimated glomerular filtration rate of ≥90 ml/min per 1.73 m^2 (normal or high), stage 2 to 60-89 ml/min per 1.73 m^2 (mildly decreased), and stage 3a to 45-59 ml/min per 1.73 m^2 (mildly to moderately decreased). Stages are based on the Clinical Practice Guideline of Kidney Disease: Improving Global Outcomes (KDIGO) (Kellum, J.A., et al., Kidney International Supplements, 2(1), 1-138).

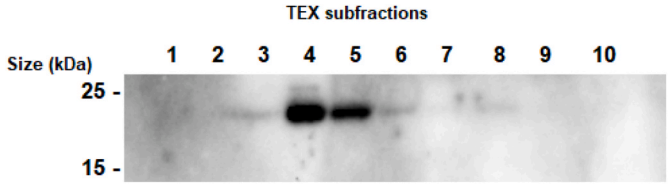

Fig. 3. Western blot showing the distribution of CD9 level in TEX subfractions. Subfractions 3-6 showed highest levels of CD9. These fractions correspond to the density 1.03–1.11 (Table 2).

3.4. Effect of colchicine on hs-CRP and IL-6

Hs-CRP was significantly lower in patients treated with colchicine (median 0.80 mg/L) compared to placebo (median 1.34 mg/L) (difference −0.54 mg/L, 95% CI -0.58 to -0.12 mg/L; $p < 0.005$) (Fig. 5). IL-6 levels were lower in patients treated with colchicine (median 2.07 ng/L) compared to placebo (median 2.59 ng/L), although this was not statistically significant (difference -0.52 ng/L, 95% CI -0.74 to 0.03 ng/L; $p = 0.076$) (Fig. 5). Levels of hs-CRP correlated significantly with IL-6 levels in patients treated with colchicine ($r = 0.40$, $p < 0.005$) or placebo ($r = 0.55$, $p < 0.005$). Hs-CRP levels did not correlate with EV NLRP3 protein levels in both treatment groups. IL-6 levels correlated with EV NLRP3 protein levels in patients treated with colchicine ($r = 0.170$, $p = 0.044$) (Supplemental Fig. 3).

4. Discussion

In this LoDoCo2 biomarker substudy, we showed *in vitro* that NLRP3 inflammasome stimulation leads to an increase in EV NLRP3 protein levels. *In vivo*, in patients with chronic coronary disease, EV NLRP3 protein levels were lower in those treated for a year with colchicine. Lastly, colchicine led to a reduction in hs-CRP that is not related to EV NLRP3 protein levels.

Table 2
Distribution of NLRP3 protein in density sub-fractions of the TEX precipitate after density gradient centrifugation.

TEX density gradient subfractions	NLRP3 protein in pg/mL	Density in g/mL
TEX 1	Not detectable	1.02
TEX 2	0.74	1.02
TEX 3	9.06	1.03
TEX 4	18.3	1.04
TEX 5	15.1	1.06
TEX 6	6.3	1.11
TEX 7	2.9	1.12
TEX 8	4.49	1.13
TEX 9	1.41	1.16
TEX 10	Not detectable	1.32

Highest concentrations of NLRP3 protein were observed in the 3-6th subfraction. Western Blot showed highest levels of CD9, which is an EV specific protein, in the same subfractions (Fig. 3). These results show that NLRP3 is indeed expressed in EVs.
EV, extracellular vesicle; IL-6, interleukin-6; NLRP3, nucleotide-binding oligomerization domain-, leucine-rich repeat-, and pyrin domain-containing protein 3; NA, not available.

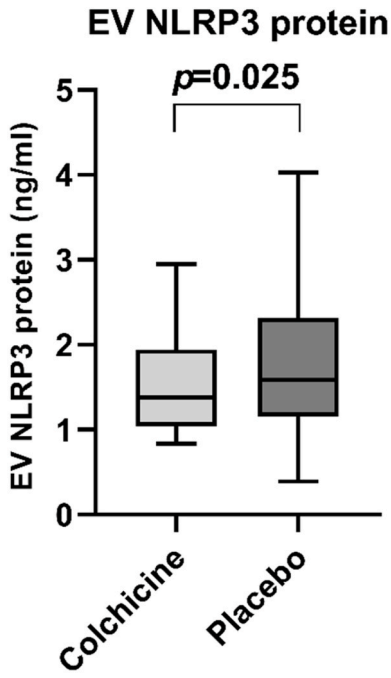

Fig. 4. Extracellular vesicle NLRP3 protein levels after one-year treatment with colchicine or placebo.
NLRP3 protein levels in EVs in patients treated with colchicine compared to placebo. Boxplots represent medians with inter quartile range and whiskers show 10-90th percentile. EV, extracellular vesicle; NLRP3, nucleotide-binding oligomerization domain-, leucine-rich repeat-, and pyrin domain-containing protein 3.

4.1. In vitro NLRP3 inflammasome stimulation

In vitro assays using LPS, ATP and other activators are widely used to investigate stimulation of the NLRP3 inflammasome [6,23–26]. These *in vitro* studies showed that NLRP3 inflammasome stimulation leads to the release of mature IL-1β [6,23,24]. Our findings reveal that NLRP3

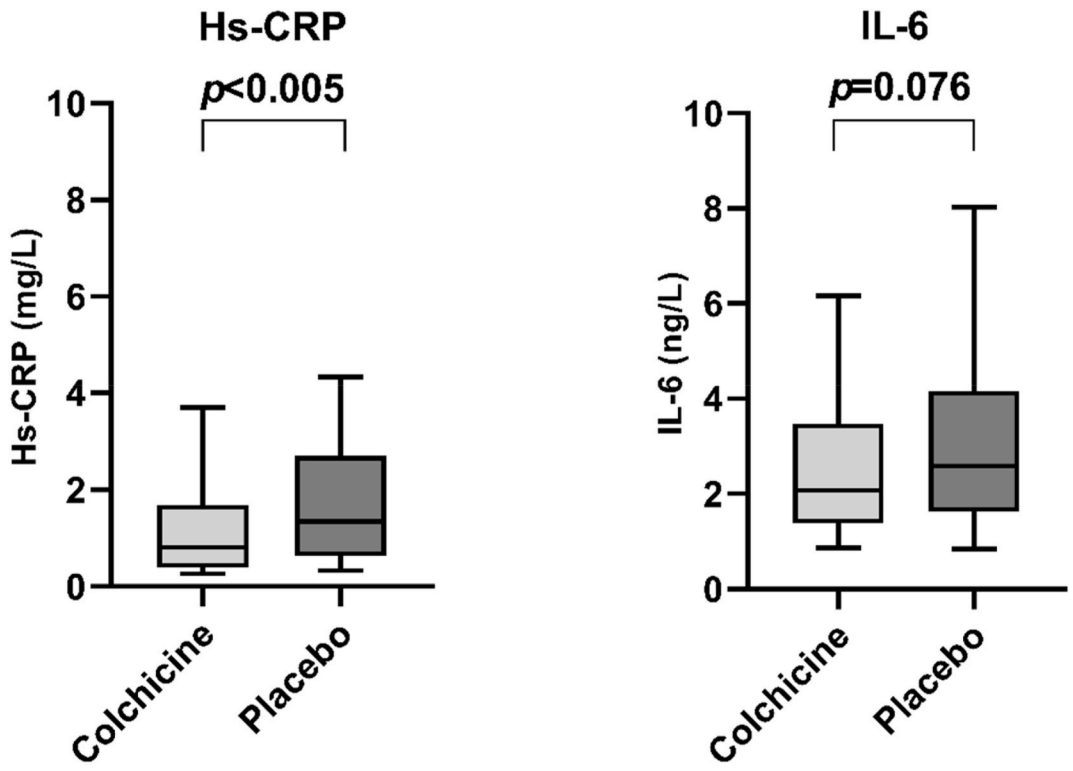

Fig. 5. Hs-CRP and IL-6 levels after one year treatment with colchicine or placebo.
Serum hs-CRP and IL-6 levels in patients treated with colchicine compared to placebo. Boxplots represent medians with inter quartile range and whiskers show 10–90th percentile. Hs-CRP, High sensitivity C-reactive Protein; IL-6, interleukin-6; NS, not significant.

inflammasome stimulation *in vitro* also leads to higher EV NLRP3 protein levels. Since NLRP3 protein is an NLRP3 inflammasome component, this may indicate that EV NLRP3 protein levels reflect the status of the NLRP3 inflammasome in the parent cells. Stimulated NLRP3 inflammasome increases mature IL-1β levels and colchicine attenuates this release [26,27]. IL-1β is also released by the activation of other inflammasomes and cleavage of intracellular pro-IL-1β can be independent of inflammasomes [15]. Therefore, EV NLPR3 protein levels may provide a more specific indicator of NLRP3 inflammasome activity. Unfortunately, we were not able to investigate the effects of colchicine on *in vitro* EV NLRP3 protein levels since colchicine in combination with LPS and ATP stimulation significantly reduced cell viability.

4.2. NLRP3 inflammasome in atherosclerosis

The involvement of the NLRP3 inflammasome and its downstream cytokines in the development and progression of atherosclerosis was described over a decade ago [28]. Since then, multiple signals (e.g. cholesterol crystals, oxidized LDL-cholesterol, calcium phosphate crystals) present in the atherosclerotic lesions were shown to activate the NLRP3 inflammasome and start a vicious circle of NLRP3 inflammasome activation and infiltration of leukocytes that leads to chronic vascular inflammation that drives detrimental atherosclerotic progression [8,9].

4.3. NLRP3 components and extracellular vesicles

The NLRP3 inflammasome consists of three components; 1) NLRP3, 2) apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC), and 3) caspase-1 [10]. Several *in vitro* studies have shown components of the NLRP3 inflammasome in secreted EVs [16,29]. Stimulated macrophages and dendritic cells release vesicles containing NLRP3 protein and caspase-1 [16,17,30]. Furthermore, EVs were associated with the release of both the pro- and mature form of IL-1β and ATP is known to activate rapid vesicle-mediated protein secretion from human macrophages, including caspase-1 and ASC [31–34]. The NLRP3 inflammasome is expressed in human inflammatory cells. Since NLRP3 protein is a NLRP3 inflammasome component, this indicates that EV NLRP3 protein levels reflect the status of the NLRP3 inflammasome in circulating immune cells and other cells and regions (atherosclerotic plaques, endothelial cells) where NLRP3 inflammasome mediated signaling is present. Along with immune cells, platelets also express the NLRP3 inflammasome [35]. Several recent *in vitro* studies showed relevant effects of colchicine on platelets and serum samples also contain a large proportion of EVs derived from platelets [36–38]. Therefore, one could hypothesize that platelet-derived EVs could also be a source of EV NLRP3 protein. In the current study, however, we did not observe EV NLRP3 protein release following platelet activation and full aggregation. Based on these results, we therefore consider it unlikely that platelets are a source of EV NLRP3 protein.

We were not able to investigate if EV NLRP3 protein has relevant physiological effects on other cells or if it solely reflects completed NLRP3 inflammasome signaling. However, released inflammasome particles, and inflammasome derived exosomes have been described to have the ability to act as danger signals to increase inflammation in surrounding macrophages [16,39,40].

4.4. NLRP3 in coronary disease and the effects of colchicine on inflammatory markers

The finding that EV NLRP3 protein levels are reduced in patients with chronic coronary disease that were treated with colchicine indicates that inhibitory effects on the NLRP3 inflammasome might contribute to the atheroprotective effects of colchicine in coronary disease.

Additional analyses of the CANTOS population revealed a residual risk for cardiovascular events that is associated with higher levels of IL-18 and IL-6 [41]. NLRP3 inflammasome inhibition may lead to the

attenuation of both IL-1β and IL-18 levels [42]. This contributes to the hypothesis that NLRP3 inflammasome inhibition, compared to selective targeting of IL-1β with canakinumab, has the potential to more effectively reduce cardiovascular events in patients with established coronary disease. However, this remains to be proven [41].

Data on direct measurements of NLRP3 inflammasome components such as NLRP3 protein in relation to atherosclerosis and coronary disease are limited. NLRP3 expression and downstream cytokines were increased in peripheral blood mononuclear cells and monocytes of patients with coronary disease compared to healthy controls [43,44]. Furthermore, lower levels of NLRP3 are associated with less severe angiographically assessed coronary disease [44].

Higher hs-CRP and IL-6 levels are associated with an increased risk for cardiovascular events [45,46]. CANTOS included patients with a history of myocardial infarction and a baseline level of hs-CRP above 2 mg/L [3]. Cardiovascular benefits of canakinumab were largest among those who achieved lowest levels of hs-CRP and IL-6 [47,48]. We previously showed a reduction in hs-CRP and IL-6 following 30 days of colchicine treatment in patients with chronic coronary disease and hs-CRP above 2 mg/L [49]. In the current study, with no hs-CRP criteria on inclusion, we also observed a reduction in hs-CRP. We were not able to investigate the relation between colchicine induced hs-CRP reduction and the risk of cardiovascular events.

Here, we demonstrate that colchicine reduces EV NLRP3 protein levels in patients with chronic coronary disease. The NLRP3 inflammasome is a pivotal driver of atherosclerosis [28]. Its inhibition by colchicine may contribute to the risk reduction of cardiovascular events as observed in the COLCOT and LoDoCo2 trials [4,5]. However, the relation between EV NLRP3 protein levels and clinical outcomes is not clear and colchicine affects a broad spectrum of inflammatory proteins [50]. How NLRP3 inflammasome inhibition contributes to the atheroprotective effects of colchicine therefore remains a topic for further investigation.

4.5. Study limitations

The EV isolations and measurements of NLRP3 were performed in a research setting since, at this stage, no standardized clinical assays exist. The limited sample size and follow-up of one year did not allow us to investigate the relation between EV NLRP3 protein levels and clinical outcomes. Although the significant reduction of EV NLRP3 strongly suggests an effect of colchicine on the NLRP3-mediated signaling pathway, the absolute difference seems small. It is, however, in line with other studies that showed similar effects of colchicine treatment on circulating IL-6 and other inflammatory proteins involved in the NLRP3 inflammasome pathway [50]. The larger absolute effect on hs-CRP (-40%) suggests that colchicine is not only inhibiting the NLRP3 inflammasome but also other inflammatory pathways and confirms previous findings [49,50]. Lastly, in 5% of patients we were unable to obtain blood samples. This was equally distributed between treatment groups.

4.6. Conclusion

Colchicine leads to a reduction of EV NLRP3 protein levels. Whether this explains the reduction in cardiovascular events in patients with chronic coronary disease remains to be determined.

Clinical trial registration

https://www.anzctr.org.au/;ACTRN12614000093684.

Financial support

Supported by the Withering Foundation the Netherlands, the Netherlands Heart Foundation, the Netherlands Organization for Health Research and Development, and a consortium of Teva, Disphar, and Tiofarma. MJMS. and LT were funded on a Dutch Heart Foundation Dekker grant (grant 2017T067). DP.dK and LT were supported by a grant of the Dutch Heart Foundation, CVON 2017-05 pERSUASIVE. The drug was supplied free of charge by TioPharma (Oud-Beijerland, the Netherlands). The funders and drug supplier had no role in study design, data collection and analysis, preparation of the manuscript or decision to publish.

CRediT authorship contribution statement

Max J.M. Silvis: Conceptualization, Methodology, Writing – original draft, Writing – review & editing, performed primary research, designed the research, Visualization, of results, wrote the manuscript, revised the manuscript. **Aernoud T.L. Fiolet:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, performed primary research, designed the revised the manuscript. **Tjerk S.J. Opstal:** Writing – original draft, Writing – review & editing. **Mirthe Dekker:** Writing – review & editing, revised the manuscript. **Daniel Suquilanda:** Investigation, performing in vitro experiments, revised the manuscript. **Minka Zivkovic:** Investigation, performing platelet activation experiments, revised the manuscript. **Michiel Duyvendak:** Resources, study medication, revised manuscript. **Salem H.K. The:** Revised the manuscript. **Leo Timmers:** Revised the manuscript, Supervision. **Willem A. Bax:** Revised the manuscript, Supervision. **Arend Mosterd:** Conceptualization, revised the manuscript, Supervision. **Jan H. Cornel:** Conceptualization, revised the manuscript, Supervision. **Dominique P. V. de Kleijn:** Conceptualization, Methodology, creation of models. Writing - original draft, revised the manuscript, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The help of the research personnel (Ms Hanneke Bakker, Ms Petra Bunschoten, Ms Marjan van Doorn, Ms Coriet Hobe, Ms Hilde Giezen) at the involved sites and the laboratory staff (Ms Claire Frissen, Ms Daniek Kapteijn & Ms Qiu Ying van de Pol) was highly appreciated.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2021.08.005.

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