



Methotrexate Inhibits Proliferation but not Proteoglycan Synthesis or Glycosaminoglycan Hyperelongation in Human Vascular Smooth Muscle Cells

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Abstract

Objectives: Atherosclerosis is a disease process involving the early deposition of lipids in the vessel wall trapped by modified proteoglycans and subsequently a chronic inflammatory process leading to the clinical events. MTX has been chosen to study the potential efficacy of an anti-inflammatory agent in preventing atherosclerosis and secondary cardiovascular disease in the cardiovascular inflammation reduction trial (CIRT).

Methods: We have investigated cell proliferation and growth factor stimulated proteoglycan synthesis in vascular smooth muscle (VSMC) to assess some of the direct effects of MTX. Experiments were conducted in cultured human VSMC. Proliferation was assessed by the gold standard technique of cell counting and proteoglycan synthesis by ³⁵S radiol sulfate incorporation and size analysis by SDS PAGE.

Key findings: MTX had a concentration-dependent inhibitory effect on serum stimulated VSMC proliferation with a maximum and total inhibitory effect at 10 μ M. Thrombin, platelet-derived growth factor and transforming growth factor beta stimulated proteoglycan synthesis and increased the size of the biglycan molecules but MTX (10 μ M) had no effect on any of these responses.

Conclusions: The outcome of a trial with MTX will reflect the potential of targeting inflammation for the prevention of atherosclerosis and it remains an interesting proposition to evaluate the effects of a "proteoglycan inhibitor" on atherosclerosis.

Keywords: Biglycan; Inflammation; Atherosclerosis; CIRT trial; Cardiovascular disease

Introduction

Cardiovascular disease is the largest single cause of premature mortality in developed countries and its major underlying pathology is atherosclerosis [1-3]. There are several hypotheses as to the aetiology of atherosclerosis of which the most prominent are the response to retention [4,5] and response to injury [6] where the latter was later modified to encompass inflammation [1,3,7]. Atherosclerosis develops slowly in the vessel wall of medium to large arteries and the subsequent rupture of an atherosclerotic plaque precipitates the occlusion and ischemia presenting as heart attack or stroke [8,9]. Inflammation has been the outstanding theme of most investigations for the last decade with atherosclerosis being routinely presented as a chronic inflammatory disease [1,3]. There are issues in relation to the balance of the contribution of inflammation to the development of atherosclerosis in humans and animal models [10] whereby the inflammatory component is considerably enhanced in animal models whereas the lipid binding by modified proteoglycans is important in the early stage of human atherogenesis [11,12]. Although much research has characterised the role of inflammation in the generation of atherosclerosis, few studies have been able to demonstrate that preventing inflammation can block the development of atherosclerosis.

With the imperative to test the validity or voracity of the inflammation hypothesis of atherosclerosis in a human trial and in the absence of a novel, well-characterised anti-inflammatory agent, the Cardiovascular Inflammation Reduction Trial (CIRT) was initiated based on the use of low dose methotrexate (MTX) (formerly known as amethopterin) [13]. MTX has been available for more than half a

century. It is an anti-metabolite and anti-folate drug which is used to treat cancer and autoimmune diseases including rheumatoid arthritis. MTX was investigated a decade ago in the context of a search for drugs to coat stents and reduce restenosis following angioplasty [14]. A recent systematic review shows that MTX has a mild beneficial impact on cardiovascular disease and mortality in people with chronic inflammatory conditions but there is no information available on the potential mechanisms of this outcome [15]. CIRT aims to test whether or not low-dose MTX will reduce rates of recurrent myocardial infarction, stroke, and cardiovascular death among stable coronary artery disease patients with Type 2 diabetes or metabolic syndrome being clinical conditions of enhanced or elevated inflammation [13]. CIRT is a randomized, double-blind, placebo-controlled trial seeking to enrol 7,000 men and women from North America to study the role of an anti-inflammatory agent in the secondary prevention of cardiovascular disease.

The major tissue involved in atherosclerosis is the vascular smooth muscle cell (VSMCs) of the vessel wall where plaque development

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occurs. The properties of VSMCs associated with atherosclerosis have been extensively explored [16-18]. These properties include cell migration and proliferation as it relates to the development of a neointima or the formation and stability of the plaque and the synthesis and secretion of proteoglycans where the latter relates to the binding and trapping of atherogenic lipoproteins by hyperelongated glycosaminoglycan (GAG) chains on the proteoglycan, biglycan in the vessel wall according to the response to retention hypothesis [5,19-22]. It is also interesting that in one of the most widely used models of atherosclerosis, the high fat fed ApoE^{-/-} mouse model, we have demonstrated that aortic smooth muscle cells from these mice compared to control wild type mice, secrete biglycan molecules with hyperelongated GAG chains that may be contributing to the lipid deposition [23]. Although MTX has been available for many years, it has not been prominently considered in the cardiovascular context and there have been very few studies of the action of MTX on the properties of VSMCs associated with atherosclerosis [14].

It should be interesting to determine if MTX has direct actions on VSMCs that if replicated *in vivo* could modify the development and progression of atherosclerosis. We have investigated the direct effect of MTX on serum-stimulated VSMC proliferation and on growth factor stimulated biglycan synthesis including GAG chain hyperelongation in human VSMCs.

We found that MTX caused concentration-dependent inhibition of serum-stimulated VSMC proliferation. At concentrations which completely inhibited proliferation, MTX had no effect on growth factor stimulated biglycan synthesis or GAG hyperelongation in human VSMCs. To the extent that the *in vitro* data showing that MTX had no effect on biglycan synthesis can be extrapolated to the *in vivo* context, this result isolates the outcome of the CIRT trial to an evaluation of the role of inflammation in the development of human atherosclerosis and its consequences.

Materials and Methods

Materials

Dulbecco's Modified Eagle Medium (DMEM) was from Invitrogen Corporation, USA. Foetal bovine serum (FBS) and penicillin-streptomycin-fungizone solution, PDGF-BB and other standard reagents were purchased from Sigma-Aldrich Australia. Carrier-free [³⁵S]-Sulfate was from MP Biomedicals, USA. Cetyl pyridinium chloride (CPC) was from Unilab Chemicals and Pharmaceuticals, Mumbai, India. Rainbow [¹⁴C] methylated protein molecular weight markers were from Amersham Pharmacia Biotech, USA. Methotrexate was also obtained from Sigma-Aldrich, Australia.

Cell culture of human VSMCs

Human vascular smooth muscle cells (VSMCs) were obtained from discarded sections of saphenous veins from patients undergoing coronary artery bypass grafting at the Alfred Hospital; the acquisition of the vessels was approved by the Alfred Hospital Ethics Committee [24]. Cells were cultured in DMEM (5.6 mmol/L glucose, 10% FBS and 1ml penicillin/streptomycin at 37°C in 5% CO₂) in 75 cm² Corning cell culture flasks (Sigma-Aldrich, MO, USA).

Cell culture protocol for proliferation assay

Human VSMC were seeded in DMEM supplemented with 10% FBS at 5.5×10³ cells/cm² into 30 mm diameter tissue culture dishes. The culture medium was replenished until cells reached 50% confluence. Four dishes were then trypsinised and basal cell number

was determined using Z2 Coulter Particle Analyser (Day 0). The remaining dishes were treated with DMEM (0.1% FBS) and (5%FBS) in the presence and absence of MTX (0.1 – 10 μM) and incubated at 37°C in 5% CO₂ for 3 days. As a vehicle control, 0.1% DMSO was added to 0.1% and 0.5% FBS, DMEM containing dishes. To determine the effect of methotrexate on proliferation of VSMC between days 0 and 3, cells were harvested with trypsin from all dishes and counted using Z2 Coulter Particle Analyser. Results are expressed as mean ± SEM of cell count from 4 separate dishes.

Quantitation of proteoglycan synthesis

For proteoglycan experiments, VSMCs between passages 15 – 22 were subcultured in 24 well plates at a density of 40,000 cells/well and grown to confluency then rendered quiescent by serum deprivation for 48 h in DMEM (5 mM glucose, 0.1% FBS and 1 ml penicillin/streptomycin). Media from the cells containing 50μCi/ml of [³⁵S]-sulfate in the presence and absence of MTX (10μM), with either thrombin (10 U/ml), platelet derived growth factor (PDGF) (50 ng/ml) or TGF-β (2 ng/ml) for 24h were harvested and added to tubes containing protease inhibitors (1M 6-amino caproic acid and 50mM benzamidine hydrochloride). Radiolabel incorporation into proteoglycans were measured by CPC precipitation assay, as describe previously [22,25].

SDS-PAGE analysis of proteoglycan size

Proteoglycans labelled with [³⁵S] sulfate were prepared for SDS-PAGE by isolation through DEAE-Sephacel anionic exchange mini columns. Samples were added to pre-equilibrated columns and then washed extensively with low salt buffer (8 M urea, 0.25 M NaCl, 2 mM disodium EDTA, 0.5% Triton X-100). Proteoglycans were eluted with high salt buffer (8 M urea, 3 M NaCl, 2 mM disodium EDTA, 0.5% Triton X-100). Equal counts of proteoglycans (20,000 - 50,000 cpm) were precipitated by ethanol solution (1.3% potassium acetate in 95% ethanol) chondroitin sulfate was added as a cold carrier. Samples were suspended in 20 μl of buffer (8 M urea, 2 mM disodium EDTA, at pH 7.5) to which 20 μl of sample buffer (0.5 M Tris-HCl pH 6.8, 10% SDS, 50% glycerol, 2-mercaptoethanol, and 0.1% bromophenol blue) were added. Radiolabeled proteoglycans were separated on gradient separating gel with 4-13% acrylamide separating gels and 3% stacking gels at 60-75 V overnight. A radiolabeled [¹⁴C] protein molecular weight marker was run simultaneously. Processed and dried gels were exposed to an imaging plate (Fujifilm BAS-MS 2040 imaging plate) for approximately 4 days. Images were developed on a phosphorimager (Fujix BAS 1000 image plate scanner) and viewed using imaging software (Fujifilm Multi-Gauge).

Statistical analyses

Data is shown as the mean ± standard error of the mean of two or more independent experiments, unless stated otherwise. Experiments were analysed by one-way ANOVA. Results were considered significant when the probability was less than 0.05 (*p < 0.05) and 0.01 (**p < 0.01). For cell counting experiments, statistical analysis was performed using one-way ANOVA, ## P<0.01 (0.1 % FBS vs 5 % FBS) and ** P < 0.01 (5 % FBS vs MTX) as well as for proteoglycan experiments, ## P<0.01 (Basal vs Agonist).

Results

Our aim was to assess the impact of the anti-inflammatory agent, MTX, on several atherogenic responses of human VSMCs being cell proliferation and growth factor stimulated proteoglycan production

and hyperelongation of GAG chains on biglycan. The experiments and methods which support and underlie these investigations have been described in multiple publications and applied to the present studies of the effects of MTX on VSMCs [1,5,6,26-31]. We initially determined the effect of MTX on the proliferation of VSMCs assessed by the gold standard method of cell counting [32]. Cell proliferation was stimulated by serum (5%) to produce a response which was not specific to any growth factor. Cells seeded at low density proliferated well in serum (5%) with an increase in total cell number of almost 3 fold in 3 days Figure 1. Cells moved to a low concentration of serum (0.1%) showed a small amount of ongoing proliferation over the 3 days period Figure 1. Cells growing in serum (5%) were also exposed to a range of concentrations of MTX (0.1–10 μ M) and cells were counted after 3 days. Visual examination of cultures showed only a small amount of apparent toxicity (floating cells) as most cells remained attached to the matrix. MTX treatment resulted in a concentration-dependent inhibition of serum-stimulated cell growth Figure 1. Marked inhibition (over 50%) of the increase in cell numbers was apparent at the lowest concentration of MTX tested (0.1 μ M) and at higher concentrations a classic dose dependent inhibition was observed with a plateau representing complete inhibition of serum-stimulated cell proliferation observed at 10 μ M MTX Figure 1.

We then assessed the effect of MTX on proteoglycan synthesis in VSMCs [33-35]. Radiosulfate incorporation is an aggregate measure reflecting incorporation of label into sulfated GAG chains and also representing an increase in core proteins (mostly biglycan) in which case more serine initiation sites are formed due to more biglycan synthesis [19]. Cells were pre-treated with MTX (10 μ M), then stimulated with a G protein coupled receptor (thrombin) [29,36], protein tyrosine kinase

(PDGF) [37,38] or protein serine/threonine kinase receptor (TGF- β) [5,30] agonists for 24h in the presence of radiosulfate. The media was assessed for secreted proteoglycans isolated by the CPC precipitation technique and quantitated by liquid scintillation counting [22] and the balance of the culture media was purified by ion exchange chromatography, concentrated then analysed by SDS-PAGE. All three agonists stimulated an increase of more than two-fold in radiosulfate incorporation into secreted proteoglycans Figure 2A. MTX (10 μ M) had no effect on the basal rate of incorporation of radiosulfate into total secreted proteoglycan Figure 2A and also had no effect on the extent of radiosulfate into secreted proteoglycans from VSMCs treated with each of the three agonists Figure 2A. Each of the three agonists stimulated an increase in the size of biglycan secreted by VSMCs and the increase in size approximately mimicked the increase in radiosulfate incorporation noted in the histogram in Figure 2A which is not necessarily a linked response as the signalling pathways share similarities but also differences [38,39]. The size of the biglycan molecules can alter due to changes in the size of the GAG chains and this was assessed by SDS-PAGE [19]. MTX had no effect on the size of biglycan secreted from untreated VSMCs and MTX also had no effect on the response to each of the three agonists to increase the size of secreted biglycan molecules Figure 2B. Thus, at a concentration of MTX (10 μ M) which totally inhibited serum-stimulated proliferation of VSMCs there was no effect on biglycan synthesis as both radiosulfate incorporation into total secreted proteoglycans and the hyperelongation of GAG chains on cells treated with thrombin, PDGF or TGF- β were unaffected. These data not only demonstrate the absence of an effect of high concentrations of MTX on biglycan synthesis and GAG hyperelongation in VSMCs but also demonstrate the differences between the signalling pathways controlling cell proliferation and biglycan synthesis.

Discussion

We examined the direct effect of MTX on atherogenic properties of VSMCs [26,40,41]. MTX caused concentration-dependent inhibition of serum-stimulated VSMC proliferation but at a concentration which completely inhibited proliferation it had no effect on biglycan synthesis or GAG hyperelongation stimulated by thrombin, PDGF or TGF- β in human VSMCs. These results demonstrate the potential for MTX to have effects on the behaviour of VSMCs in the processes of atherogenesis but those actions do not include an action on proteoglycan synthesis.

We have previously demonstrated that gingerol inhibits TGF- β stimulated proteoglycan synthesis without inhibiting TGF- β signalling assessed as Smad transcription factor phosphorylation [42] and genistein inhibits PDGF effects on versican synthesis without blocking PDGF receptor phosphorylation [41]. So although an effect of MTX was conceivable, it did not occur, notwithstanding that MTX was highly effective in blocking VSMC proliferation.

Atherosclerosis occurs due to the slow formation of a neointima in atherosclerosis-prone vessels followed by an early stage involving the trapping of atherogenic lipoproteins by modified proteoglycans with hyper elongated GAG chains [12,43] and the resulting modified lipoprotein particles act as immunogens in the vessel wall to initiate a long slow chronic inflammatory process which leads to the formation of atherosclerotic plaques. The rupture of vulnerable atherosclerotic plaques precipitates the clinical event of a heart attack or stroke [1,3,7]. The atherosclerotic process is different in rodents and humans in terms of the temporal aspects and also the balance between the early matrix-dependent phase and the late inflammatory phase [10]. In rodents the process is accelerated and dominated by the inflammation phase but in humans the early proteoglycan-dependent phase is more pronounced

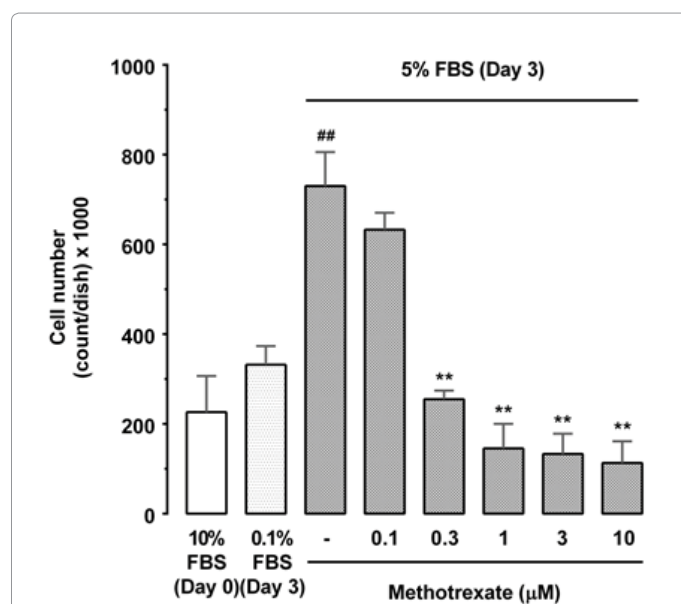
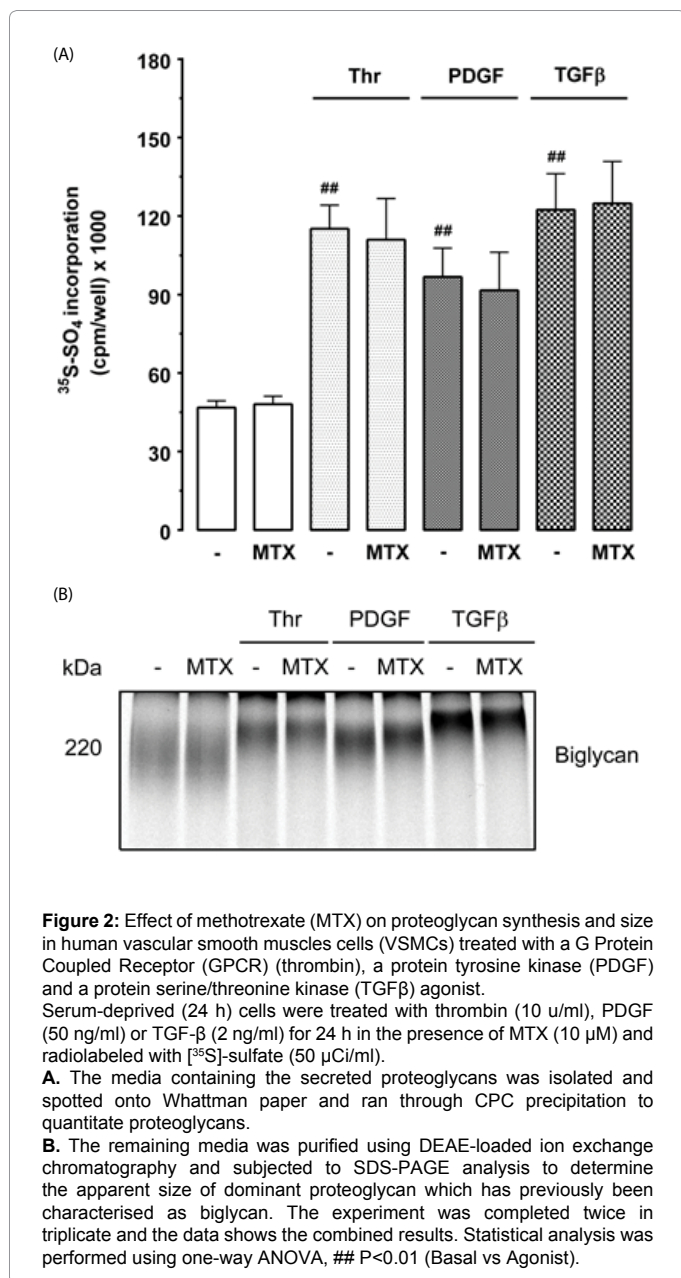


Figure 1: Effect of methotrexate on serum-stimulated proliferation of human vascular smooth muscle cells assessed by cell counting. Cells were seeded at low density in 30 mm diameter plates and grown in DMEM with 10% fetal calf serum (FBS). After 3 days cells were transferred to 0.1% FBS medium or 5% FBS medium with or without Methotrexate (0.1–10 μ M). Cells in all dishes were examined visually then counted after 3 days. The experiment was repeated three times with near identical results. Results are expressed as mean \pm SEM of cell count from 4 separate dishes. Statistical analysis was performed using one-way ANOVA, ## $P < 0.01$ (0.1% FBS vs 5% FBS) and ** $P < 0.01$ (5% FBS vs Methotrexate).



before the inflammatory process leading to the clinical consequences [11]. The CIRT trial is investigating the anti-inflammatory role and potential protective action of MTX which is targeted at the secondary prevention of disease events [13]. Thus, the trial is focussed on the most likely target to obtain a beneficial effect of MTX. Modified proteoglycans probably also play a role in later stages of atherosclerosis [44] by continuing to manifest the trapping of lipoproteins but this is likely to be less important than the early role which initiates the process of atherosclerosis [12]. Our results demonstrating that MTX has no effect on proteoglycan synthesis in human VSMCs indicates that the outcome of the trial will not be due to an action on vascular smooth muscle cell-derived proteoglycans, specifically biglycan as was investigated in this study. The corollary is that a favourable outcome will generate interest in pursuing the role of inflammation as a target for the prevention of cardiovascular disease with the aim of generating new, better, more

efficacious and safer anti-inflammatory agents. In addition, there remains a need to develop specific inhibitors of proteoglycan synthesis and specifically inhibitors of GAG hyperelongation to evaluate their effects on early and late stage atherosclerosis as has been successfully demonstrated in animal models [19,45].

It is interesting to consider and compare the role of statins in preventing adverse cardiovascular events. Statins are highly efficacious in the prevention of cardiovascular events with a maximum effect in the tightly controlled environment of a clinical trial of about 30 per cent [46-48]. Statins inhibit HMG-CoA reductase and lower plasma cholesterol [49] but they also have a range of additional anti-inflammatory and anti-oxidant effects that most likely contribute to their efficacy. We have recently established assays for the expression of the genes mediating hyperelongation of GAG chains in VSMCs and we have observed that statins have no effect on the growth factor stimulated expression of chondroitin 4-sulfotransferase-1 and chondroitin synthase-1 (unpublished observations). This data indicates that an action of proteoglycan synthesis does not contribute to the efficacy of statins in preventing cardiovascular events in humans but also validates the need to assess the effect of a proteoglycan inhibitor on cardiovascular events with the possibility that such a product may have synergistic effects when used with a statin as both drugs would have independent and complementary anti-atherosclerotic actions [50].

An interesting outcome of the current studies is that they demonstrate the dissociation of the processes of proliferation and proteoglycan synthesis. This can be difficult to explore experimentally because most agents stimulate proliferation also stimulated proteoglycan synthesis [27,30,38,51]. This is not universally the situation since TGF-β is one of the most effective stimulators of biglycan synthesis and GAG hyperelongation but it is not mitogenic for normal differentiated VSMCs [52]. Studies using xyloside as false acceptor for the initiation of GAG chains have also revealed the distinction of proliferation and proteoglycan synthesis [53]. It is potentially very useful in the search for an agent to prevent proteoglycan synthesis as an anti-atherosclerotic agent that its actions are specific for proteoglycan synthesis.

The effect of MTX on VSMC proliferation has been investigated a decade ago in the context of the evaluation of drugs as stent coatings for use in angioplasty [14]. In an experiment comparing MTX and paclitaxel, using rabbit aortic smooth muscle cells, the MTX had no effect but the paclitaxel treatment resulted in concentration dependent reduction in cell numbers [14]. Examining the *in vitro* cell culture experiment, in contrast to our experiment with cell number expansion see Figure 1, it is not clear as to the extent of proliferation that the cells were undergoing in the studies of Huang et al. [14]. These authors reported that the cells showed 100 per cent viability in the presence of MTX and only 50 per cent viability in the presence of paclitaxel so it is possible that the different experimental design – proliferation versus viability – might explain the different results. It is also noted that the inhibition of VSMC proliferation reported in our study is consistent with the marked reduction in neointimal hyperplasia reported by Huang et al. [14] in their porcine model of restenosis and neointimal hyperplasia.

Conclusion

We have demonstrated that the anti-inflammatory folate antagonist, MTX, causes concentration-dependent inhibition of serum-stimulated proliferation of human VSMCs and at a concentration which completely inhibits cell proliferation; it has no inhibitory actions on the production and secretion of biglycan synthesis stimulated by

three different diverse signalling growth factors. So far as MTX is being investigated in a clinical trial of the secondary prevention of cardiovascular events, this data if extrapolated to an *in vivo* setting would foreshadow that an action on proteoglycan synthesis will not contribute to the outcome of the index trial and it is therefore a focused evaluation of the effect of an anti-inflammatory agent in preventing cardiovascular disease.

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