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Modulation of IL-1, IL-6 and IL-18 cytokine signaling in animal models of atherosclerosis: a systematic review evaluating animal sex in preclinical research

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ABSTRACT

Targeting of IL-1, IL-6 and IL-18 signaling is a main focus of anti-inflammatory therapy development against atherosclerosis. In preclinical atherosclerosis studies, animal sex may however affect therapeutic efficacy. Evidence for this hypothesis is lacking. We therefore aimed to study sex used and influence of animal sex on therapeutic efficacy of IL-1, IL-6 and IL-18 pathway interventions in atherosclerosis animal models. Medline (PubMed) and EMBASE (OVID) were comprehensively searched and screened to identify studies investigating the effects of targeting IL-1, IL-6 and IL-18 signaling in atherosclerosis animal models. Study characteristics and plaque size data were extracted. Individual effect sizes were calculated and pooled using the random effects model. Predefined subgroup analyses for sex were conducted. From 1744 retrieved studies, 62 papers were included in this systematic review, of which 47 papers in the meta-analyses. All 47 studies used mice, of which 41 investigated inhibitory interventions and 8 stimulatory. Meta-analyses showed a significantly smaller plaque size upon inhibition of cytokine signaling (SMD: -1.5 [-1.8 to -1.2], n = 39) and a significantly larger plaque upon stimulation (SMD: 1.2 [0.3 to 2.1], n = 8). The majority of the studies included male mice. Due to the limited number of studies with females, subgroup analysis for sex could only be performed for males and revealed no differences compared to the overall analyses, suggesting that male mice are suitable for such studies. More studies with female mice are required to truly assess whether animal sex is a variable in treatment efficacy of IL-1, IL-6 and IL-18 pathway interventions in atherosclerosis.

1. Introduction

Annually, around 17.9 million people die as a result of cardiovascular diseases (CVDs), with ischemic heart disease and stroke as the most common causes (World Health Organization). The most prevalent pathology underlying many CVDs is atherosclerosis, which is caused by the buildup of lipoproteins and inflammatory cells in the vascular wall of the large arteries. Upon rupture or erosion of an atherosclerotic plaque, a vessel-occluding thrombus can be formed, causing acute cardiovascular events (ACE) (Bjorkegren and Lusis, 2022; Libby, 2021). Current treatment to reduce atherosclerosis burden is mainly aimed at lowering plasma cholesterol levels using statins or, more recently, PCSK9 inhibitors. While this has reduced the incidence of acute cardiovascular events significantly (Baigent et al., 2010; Sabatine et al., 2017), a residual inflammatory risk remains (Ridker, 2017), rendering novel insights in disease pathology and underlying mechanisms still mandatory.

To target the residual inflammatory risk, recent clinical trials focused on anti-inflammatory therapeutic strategies in high risk CVD patients. In the CANTOS trial, the therapeutic monoclonal antibody against interleukin-1β (IL-1β), canakinumab, significantly reduced the incidence of secondary cardiovascular events (Ridker et al., 2017). Colchicine, an anti-inflammatory agent (Ait-Oufella and Libby, 2024), significantly lowered the occurrence of cardiovascular events in patients with stable coronary disease in the LoDoCo trials (Nidorf et al., 2013; Nidorf et al.,

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2020). More recently, in the RESCUE trial, ziltivekimab, a therapeutic monoclonal antibody against the IL-6 ligand, reduced high-sensitivity C-reactive protein (hs-CRP) levels in patients with chronic kidney disease combined with elevated blood hs-CRP (Ridker et al., 2021).

These studies clearly show the clinical potential of anti-inflammatory therapeutic strategies to lower the inflammatory risk and prevent ACE, and justify further study of inflammatory mechanisms that underly atherosclerosis. Preclinical atherosclerosis models can aid in studying such mechanisms, as well as in investigating potential targets of intervention relevant for human disease. Preclinical animal models of atherosclerosis (Gisterå et al., 2022) include mouse (Ishibashi et al., 1993; Plump et al., 1992; Zhang et al., 1992), rabbit (Shiomi et al., 2003) and pig models (Griggs et al., 1986). Atherosclerotic mouse models are most commonly used, as mice reproduce easily and atherosclerosis develops fairly fast. Additionally, genetic modifications are relatively easy to induce. In the past decades, two widely used mouse models have been generated, being the apolipoprotein E knockout (*Apoe*$^{-/-}$) (Plump et al., 1992; Zhang et al., 1992) and the low-density-lipoprotein receptor knockout (*Ldlr*$^{-/-}$) (Ishibashi et al., 1993) mouse strains, both develop atherosclerosis upon hypercholesterolemia (Ishibashi et al., 1993, 1994; Plump et al., 1992; Zhang et al., 1992). For studies requiring a more humanized lipoprotein profile, the APOE3-Leiden mouse model or the APOE*3 Leiden.CETP transgenic model (Westerterp et al., 2006) are used. These latter mouse strains show hampered clearance of apoB-containing lipoproteins and elevated triglyceride levels, and are, in contrast to the *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ strains, responsive to lipid-lowering therapeutics such as statins (Gisterå et al., 2022; van den Maagdenberg et al., 1993). These mouse models are widely used in preclinical atherosclerosis research, but the translational potential of such preclinical studies to human disease can be hampered by strain choice and study setup, including animal sex. It is unclear whether sex of the animal affects outcome of anti-inflammatory therapy in preclinical atherosclerosis. Already in 1987, differences in atherosclerosis development in male versus female mice were reported (Paigen et al., 1987), in relation to differences in lipoprotein profiles. More recently, aged female *Ldlr*$^{-/-}$ mice were showed to have increased and altered immune cell populations in the aortic atherosclerotic plaque compared to aged male mice (Smit et al., 2024), which suggests that there may be a difference in response to inflammatory stimuli or anti-inflammatory therapy. In this systematic review, we therefore aimed to determine whether a difference in therapeutic efficacy exists between sexes of atherosclerotic animals, focusing on studies investigating interventions in the inflammatory response. We selected animal studies in which the cytokines IL-1, IL-6 and IL-18, targets of interest in recent clinical trials, were pharmacologically modulated, either via inhibitory or stimulatory strategies. The outcome of this review may contribute to the more refined design of animal experiments concerning the animal sex in preclinical atherosclerotic studies related to inflammation in atherosclerosis.

2. Methods

In this systematic review, we investigated the effects of inflammation therapeutics on plaque size in animal models of atherosclerosis, with the specific question whether therapeutic efficacy differs between male and female animals. We focused on targeting of IL-1 (including both IL-1α and IL-1β), IL-18 and IL-6 cytokine signaling. Our systematic review protocol is registered in the international prospective register of systematic reviews, PROSPERO under ID CRD42024513510, and reported following the PRISMA guidelines (Page et al., 2021a,b).

2.1. Search and study identification

To find the relevant papers, search strings were designed for both Medline (PubMed) and EMBASE (OVID). The complete search strings are displayed in Supplementary Tables 1 and 2. Literature searches were performed on November 14th, 2023.

2.2. Study selection

From the obtained records, papers occurring more than once were removed. The title and abstract of each remaining paper were screened by two independent reviewers (LD, CH, NI or IB) for the following exclusion criteria using Rayyan screening software: (1) reviews (2) human studies, (3) studies using only in vitro assays, (4) vascular remodeling models such as denudation, (partial) ligation and vein graft disease models, (5) type of study other, (6) if only non-inflammatory intervention is used, (7) commentaries, (8) case-reports, (9) protocols, (10) short communications, (11) editorial, (12) personal opinions, (13) letters, (14) posters, (15) conference abstracts, (16) dissertations, (17) thesis and graduation work, (18) other. Discrepancies were resolved by discussion with a third reviewer (LD or IB).

After the exclusion of papers based on title and abstract, the full-text document of each remaining paper was screened by two independent reviewers (LD, CH or IB) for exclusion criteria using Rayyan screening software (Ouzzani et al., 2016). These included the same criteria as for the title and abstract screening and the following additional exclusion criteria: (2) modulating molecules not being the IL-1, IL-6 and IL-18 cytokines or their receptors, (3) studies that present only genetic or epigenetic data, (4) studies that do not show atherosclerotic lesion data, (6) other exclusion criteria. In the full-text screening phase, disagreements and discrepancies were resolved by consensus after discussion with a third reviewer as well (LD or CH).

We decided post-hoc to exclude experiments (comparisons) in which genetically modified animal models were used, where the gene modification could have interfered directly with one of the investigated pathways.

Furthermore, we initially planned to assess whether the sex affects the therapeutic efficacy upon intervention. During the conduct of the systematic review we decided to post-hoc perform meta-analyses in which we determined the overall effect on plaque size for the different targets as well, mainly because no studies were available formally assessing the difference between males and females. As a consequence two criteria for exclusion that are mentioned in our protocol, are not used in practice.

2.3. Extraction of study characteristics

The following study characteristics were extracted by one reviewer (LD): author, year of publication, title, journal, language, doi, species, strain, genetic background, type of atherosclerotic model, sex, age (at disease induction, t = 0), disease induction method, type of diet, cytokines, cytokine receptors, type of intervention, type of immunomodulatory component, timing intervention (relative to disease induction), timing of outcome assessment (relative to intervention) and duration.

After publishing the protocol, we also decided for the papers included in meta-analysis to record whether the immunomodulator(s) were either inhibitory or stimulatory, and whether the immunomodulatory component was a direct modulator of the target of investigation.

2.4. Reporting quality and risk of bias assessment

Two researchers (LD, IB) independently assessed reporting quality and performed a risk of bias assessment for each included paper. The SYRCLE's RoB tool (Hooijmans et al., 2014) was used to assess the risk of bias. For the bias aspect on baseline group similarity, the researchers focused on age, sex and strain. Both assessments consist of questions where the answer "yes" indicates a low risk of bias, a "no" a high risk and a "?" an unclear risk of bias. Five reporting questions were answered: (1) is it mentioned that the experiment was randomized at any stage? (2) is it mentioned that the experiment was blinded at any stage? (3) is a power/sample size calculation reported? (4) is a conflict of interest

statement reported? (5) does the study mention a prespecified/preregistered protocol? For the reporting questions, "yes" indicates reported and "no" indicates not reported. Discrepancies were resolved by discussion.

2.5. Extraction of outcome data

Next, to be able to perform the meta-analysis, aortic atherosclerotic lesion size data of each independent comparison was collected by one reviewer (LD) and checked by a second reviewer (IB). This data included the lesion size mean, SEM or SD, the unit and sample size (Supplementary Table 6). If the SEM was extracted, the SD was calculated from these data by SEM*(SQRT(n)). In order to reduce the observed heterogeneity, we decided during conduct of this systematic review to only include data containing plaque size values on the aorta, and not atherosclerosis data at other vascular locations. For aortic plaque data, the aortic root was prioritized as location over other parts of the aorta, when applicable. Furthermore, to be included in the meta-analysis, studies in which antibody treatment was applied should have included an appropriate isotype control antibody. Similarly, for lentiviral vector derived interventions a control vector should have been included in the study. Combination treatments were not included.

If the outcome data was only presented in graphs, plotdigitizer.com/app was used to extract the data for analysis. If outcome data was missing or unclear, the steps described in Supplementary Table 3 were taken. When authors were contacted, one email and a subsequent reminder were sent. In case it was unclear whether the SD or SEM was presented, the individual data points were measured to determine whether the presented value was the SD or SEM. If the number of animals (N) was presented as a range, the individual data points were counted. If counting was not possible, the lowest value was chosen. For these and additional exceptions the detailed actions taken are presented in Supplementary Table 3.

2.6. Data analysis/synthesis

2.6.1. Overall effect on plaque size per target

Outcome data was grouped into the different targets: IL-1α, IL-1β, IL-6, IL-18, IL-1R, IL-6R. Treatment strategies targeting the NLRP3 inflammasome or caspase-1 were included in both the IL-1β and IL-18 groups, because both of these cytokines could have been affected by these interventions. Subsequently, for every target the data were grouped into inhibitory or stimulatory interventions. Similarly, data were grouped according to direct or indirect targeting. Additionally, we separately pooled the inhibition and stimulation data of all targets. We performed meta-analyses to determine the overall effect on plaque size for every group in Comprehensive Meta-Analysis (CMA) software version 4. The standard mean difference (Hedges g) and 95 % confidence interval for plaque size between the experimental groups and control groups were calculated and subsequently pooled using the random effect model. The between study heterogeneity was estimated with I-squared (I^2). We corrected for multiple use of the control group, by adjusting the sample size of the control group by dividing by the number of times the control group was used (Duque-Quintero et al., 2022).

2.6.2. Sensitivity analyses

To assess the robustness of our results, we performed sensitivity analyses for the IL-1β and IL-18 groups, because of the inclusion of the NLRP3 inflammasome and caspase-1 data in both groups. The sensitivity analyses comprised a comparison between analyses with and without the NLRP3 inflammasome and caspase-1 data to determine whether the additional data affects the effect on plaque size.

2.6.3. Sex subgroup analyses

To determine whether sex influenced therapeutic effects on lesion size, we envisioned two scenarios in which we would be able to perform a subgroup meta-analysis for sex. One scenario would require at least three independent studies in which the effects of therapy on lesion size was presented in both sexes. Another scenario required at least 10 studies using male animals, and 10 studies using female animals. Subgroup analysis were envisioned for the second scenario. During subgroup analyses a common among-study variance component across subgroups was assumed. The subgroups (male and female) were combined using fixed effects model (Mulder et al., 2024).

2.7. Publication bias

Egger's regression to identify the risk of small study effects was conducted for all outcomes containing a minimum of 15 independent studies. Because SMDs were used in meta-analyses, the precision estimate was adjusted (Zwetsloot et al., 2017).

3. Results

3.1. Study selection

Fig. 1 shows the flow chart of our study selection process. In brief; 1744 references were screened by two independent reviewers. 62 papers (Abderrazak et al., 2015; Akita et al., 2017; Bhaskar et al., 2011; Bhat et al., 2015, 2018; Chen et al., 2012, 2022; Christersdottir et al., 2019; de Nooijer et al., 2004; Denes et al., 2012; Dragoljevic et al., 2020; Elhage et al., 1998; Fidler et al., 2021; Freigang et al., 2011; Fukumoto et al., 1997; Fuster et al., 2017; Gomez et al., 2018; Hettwer et al., 2022; Hohensinner et al., 2021; Imai et al., 2011; Janssen et al., 2015; Jia et al., 2023; Jin et al., 2022; Karnewar et al., 2024; Ku et al., 2022; Li et al., 2020a; 2020b, 2021; Liu et al., 2020, 2023; Luo et al., 2020a; 2020b, 2021; Ma et al., 2023; Mallat et al., 2001; Meng et al., 2016; Orecchioni et al., 2022; Schuett et al., 2012; Schwarz et al., 2023; Shentu et al., 2021; Shimokawa et al., 1996, 2001; Spartalis et al., 2021; Takeda et al., 2006; Tenger et al., 2005; Tian et al., 2020; Tissot et al., 2013; Tous et al., 2006; Traughber et al., 2023; van der Heijden et al., 2017; Vromman et al., 2019; Wan et al., 2019; Wang et al., 2020; Wen et al., 2021; Whitman et al., 2002; Wu et al., 2022; Xie et al., 2021; Xu et al., 2021, 2023; Yalcinkaya et al., 2023; Zhang et al., 2012; Zhao et al., 2020) were included in the systematic review, of which 47 papers were included in the meta-analyses (Fig. 1). Three comparisons from three different papers (Liu et al., 2020, 2023; Orecchioni et al., 2022) were not included in the systematic review, because in these mouse models gene modifications potentially interfered with the pathways we aimed to investigate.

3.2. Study characteristics

The study characteristics of the experiments described in the 62 selected papers are displayed in Supplementary Table 4. The first papers were published in 1996 and 1997 (Fig. 2A), both describing IL-1β targeting studies in pigs. Most of the included papers were published in 2020 (Fig. 2A). In 94 % of the papers, mice were used as model for atherosclerosis, in 5 % of the papers pigs and in 2 % rabbits (Fig. 2B). Most of the papers (63 %) used only male animals and 14 % used only females for comparisons with specified sex. In two papers both males and females were used separately, while in 6 % of the papers, the groups consisted of a mix of males and females. In 13 %, the sex of the animals was not reported (Fig. 2C).

3.2.1. Characteristics of papers included in overall meta-analyses

47 papers presented all data needed regarding plaque size and were suitable to be included in the overall meta-analyses (Fig. 1). In these papers only mice were used (Fig. 3). For most of the papers, for which the age of the mice at outcome assessment was available, mouse age fell in the category "16 up to and including 23 weeks" (58 %). Three different mouse strains were used in the papers, with the *Apoe*$^{-/-}$ mice

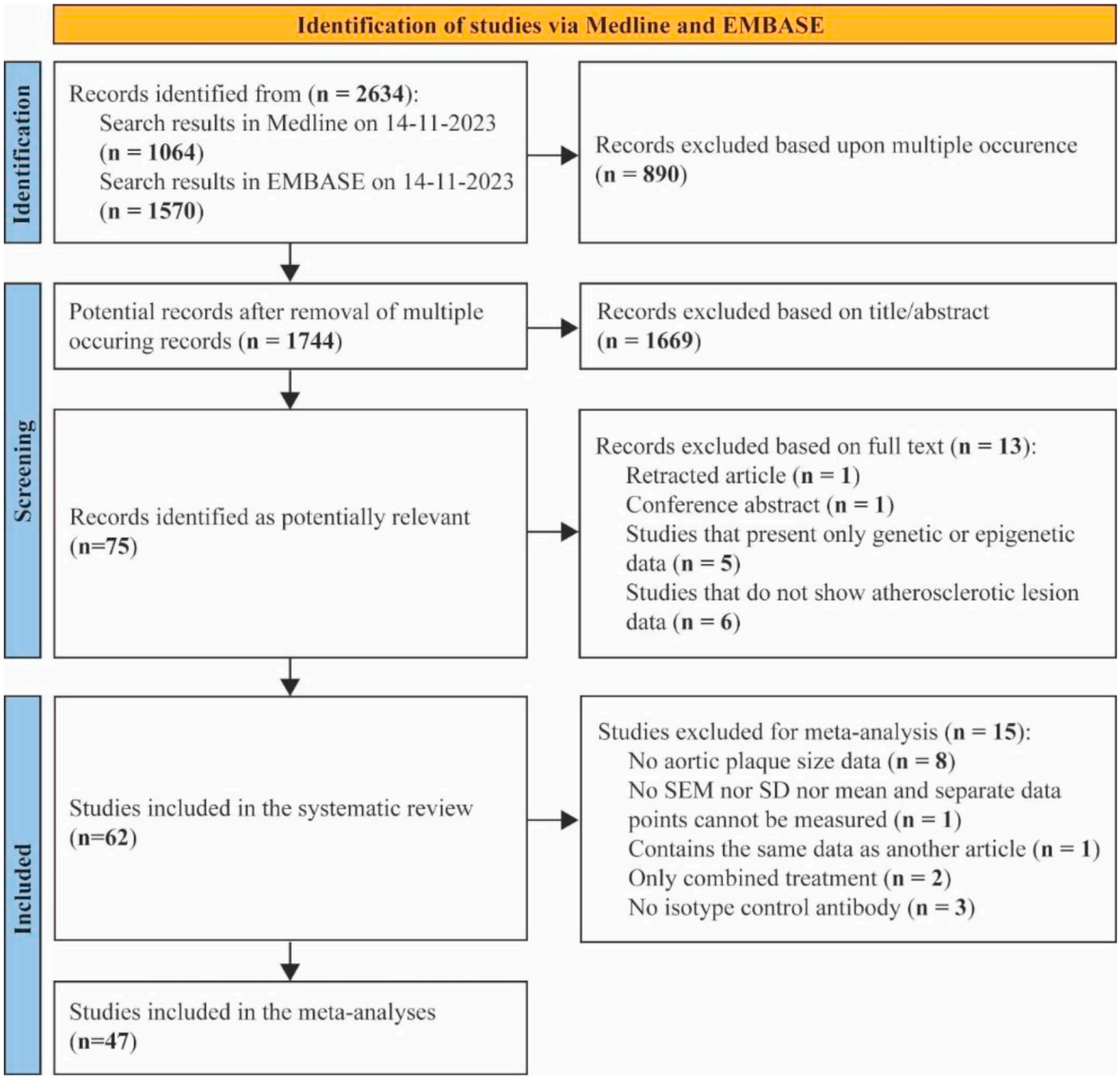

Fig. 1. Flowchart of the record selection process. Illustration of the steps for the selection of relevant studies (Page et al., 2021a,b).

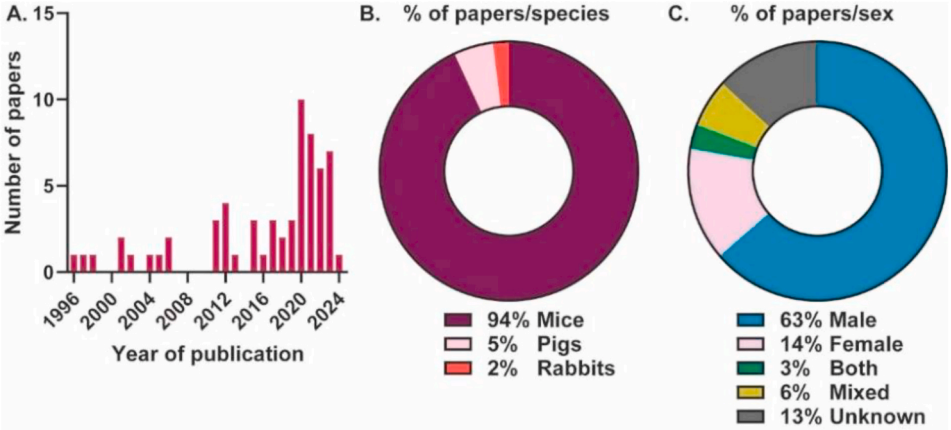

Fig. 2. Publication period of the included papers and species and sex used. A. The number of papers per publication year. The percentage of papers per B. species and C. sex.

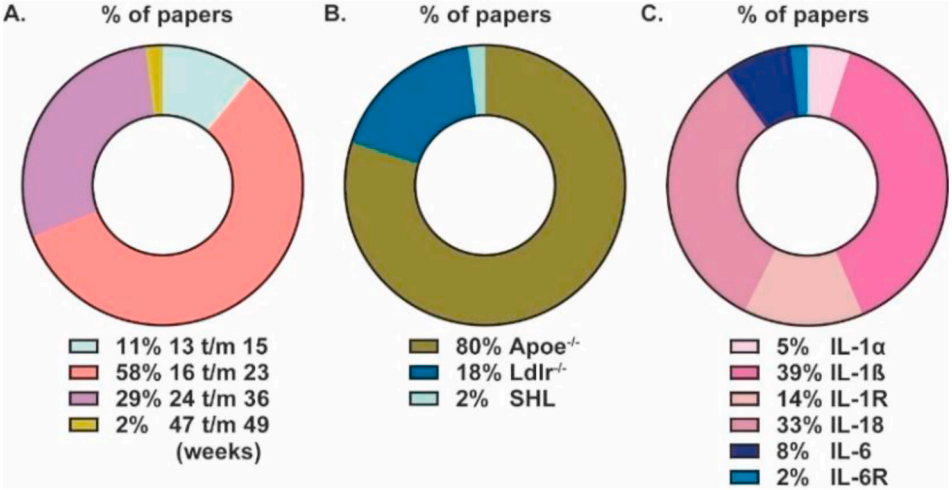

Fig. 3. Study characteristics of the papers included in the meta-analyses. The percentage of papers per A. age at outcome assessment category, B. mouse strain and C. target.

being most abundantly applied (80 % of the papers), followed by the *Ldlr*$^{-/-}$ mice (18 % of the papers). Since we were interested in IL-1, IL-6 and IL-18 signaling, we defined which cytokine or cytokine receptor was targeted in the papers. In most papers (39 %), IL-1β was targeted, followed by IL-18 (33 %). As IL-1 and IL-18 both originate from the Interleukin-1 cytokine family, we report the outcomes of IL-18 interventions directly after IL-1 interventions. In addition, some of the papers describe more than one experiment or multiple experimental groups of interest for our analyses. We therefore extracted the individual experimental data per group, resulting in a total of 73 comparisons that were obtained from the included papers for the overall analyses.

3.2.2. *Characteristics of papers included meta-analyses regarding sex*

Sex of the animals was specified in 41 papers of the 47 included in our meta-analyses. Of these 41 papers, 80 % used only male mice (n = 33), 15 % only female mice (n = 6) and 5 % used both (n = 2) sexes separately in the specified comparisons (Fig. 4A). In most of the papers, for which the age at outcome assessment was available, the age range was "16 up to and including 23 weeks" (55 %) and *Apoe*$^{-/-}$ mice were mainly used (80 %). Also, in this subset of papers, IL-1β (38 %) was most often targeted, followed by IL-18 (33 %). In Fig. 4B, we also specified the information on study characteristics per sex.

3.3. *Reporting quality and risk of bias*

The reporting quality (Fig. 5A) and the risk of bias (Fig. 5B) were assessed in all papers included in this systematic review. The detailed assessments are shown in Supplementary Table 5 and 6 Regarding the reporting quality, 47 % of the papers mentioned some form of randomization in the experiments, which entailed randomization of treatment groups or of treatment sites. In addition, 44 % described some form of experiment blinding. Only four papers reported a power/sample size calculation. A conflict of interest statement was reported in 74 % of the papers. None of the papers mentioned a prespecified/preregistered animal study protocol (Fig. 5A).

The risk of bias assessment showed that in only one paper a clear and adequate allocation sequence generation and application was described (Fig. 5B). The baseline characteristics age, sex and animal strain were generally well described and did not pose a risk of bias in 77 % of the papers. In only one of the papers allocation sequence concealment was specified, as in this paper group assignment was mentioned to be non-randomized and non-blinded. 92 % of the papers did not mention random housing of the animals during the experiment and 79 % did not mention whether the caregivers/investigators were adequately blinded during the experiment. Furthermore, it was not mentioned in any of the papers whether the outcome assessment was performed in a random fashion and 58 % did not mention blinding of outcome assessment. Incomplete outcome data was adequately addressed in 24 % of the papers and 85 % of the papers did not selectively report outcome data. In 19 % of the papers additional risks of bias were detected. For example, one paper (Bhaskar et al., 2011) compared a single concentration of a control antibody to multiple concentrations of the treatment antibody. Meng and colleagues (Meng et al., 2016) did not include an experimental method of the *in vivo* antibody treatment, only which antibodies were used, and not of the plaque staining analysis. As a last example, Xu et al. (2023) described an n-value of 3, which is very limited for atherosclerosis experiments (Daugherty et al., 2017).

3.4. *Meta-analyses*

The extracted outcome data is presented in Supplementary Table 7. Meta-analyses results are presented in Table 1 per cytokine modulation group. Direct inhibition of IL-1α led to a significantly smaller plaque size. No data was available regarding indirect inhibition, nor direct and indirect stimulation of IL-1α. Direct and indirect inhibition of IL-1β significantly decreased plaque size and direct stimulation seems to increase plaque size, although this dataset contained only one study. Indirect stimulation of IL-1β did not significantly affect plaque size. Plaque size significantly decreased after direct and indirect inhibition of the IL-1R. No data regarding stimulation of the IL-1R was available. IL-18 inhibition (both direct and indirect) significantly decreased plaque size and direct and indirect stimulation of IL-18 significantly increased plaque size. Finally, direct and indirect inhibition of IL-6 resulted in significantly smaller plaques. Remarkably, direct stimulation of IL-6 also significantly decreased plaque size, but again here, this dataset contained only one comparison. There was no data on indirect IL-6 stimulation. Regarding the IL-6R, there was only one comparison investigating the effect of direct inhibition, but this did not induce a significant effect on plaque size. No data regarding indirect inhibition of Il-6R or IL-6R stimulation was available. The overall meta-analysis for all comparisons investigating the effect of inhibition showed that plaque size significantly decreased upon inhibition as indicated by a negative standardized mean difference (-1.5 [-1.8 to -1.2]), where a value of 0 would indicate no difference in effect. All comparisons investigating the effect of stimulation, showed an increase in lesion size increased after stimulation of the pathways (1.2 [0.3 to 2.1]) (Table 1). Together, these data illustrate a pro-atherogenic effect of these cytokines, with therapeutic efficacy upon inhibiting these cytokine pathways.

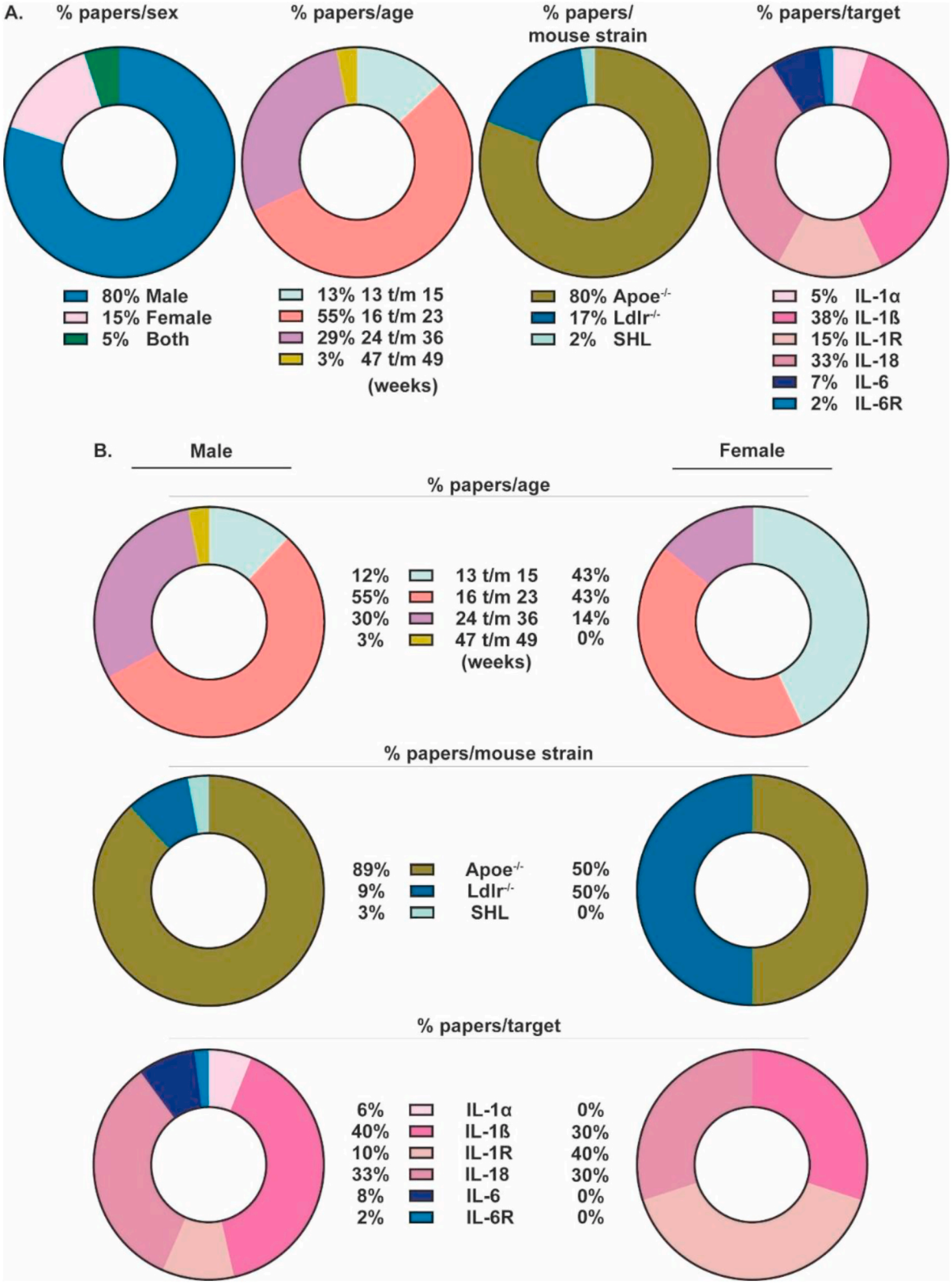

Fig. 4. Study characteristics of the meta-analyses papers with specified animal sex. A. The percentage of papers per sex category, age at outcome assessment category, mouse strain and target. B. The percentage of papers for male (left) and female (right) mice per age category, mouse strain and target.

3.4.1. Sensitivity analyses

Treatments targeting the NLRP3 inflammasome or caspase-1 can affect both the IL-1β and IL-18 pathway and were therefore included in both these treatment groups. To determine whether the NLRP3 inflammasome and caspase-1 data affects the effect estimate of the IL-1β and IL-18 groups, sensitivity analyses were performed excluding the NLRP3 inflammasome and caspase-1 comparisons (Table 2). In general, our analyses seem robust and nearly all effects remain the same, suggesting that efficacy of such interventions provide similar outcomes as direct IL-1β and IL-18 interventions. The only difference was the effect of IL-1β

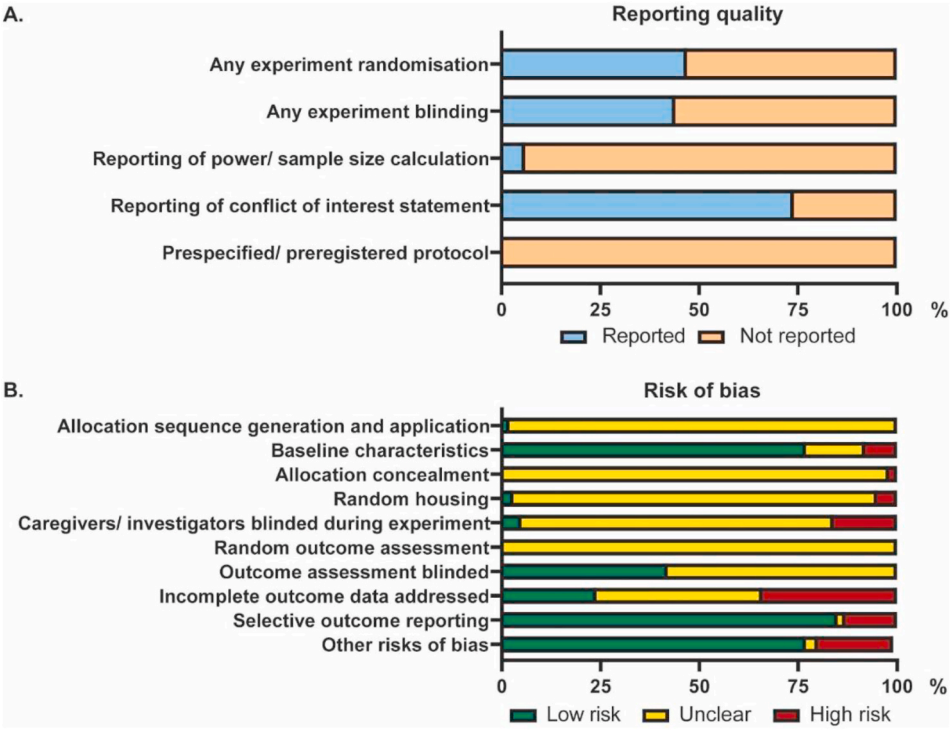

Fig. 5. Risk assessments of the 62 included papers. A. Reporting quality assessment results presented as the percentage of papers that did (blue) or did not (orange) report the specific component of the total included papers. B. Risk of bias assessment results displayed as the percentage of papers in which the specific component scores a high risk (red), unclear risk (yellow) and low risk (green) as a percentage of all papers. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

indirect stimulation. However, it has to be taken into account that the sensitivity analysis of IL-1β indirect stimulation was based on one single study.

3.4.2. *The effect of sex*

To determine the effect of the sex on lesion size we envisioned two scenarios (see methods). Regarding scenario 1; only two studies investigated the difference between the sexes. As a consequence, no formal meta-analysis is conducted. The first study, by Elhage et al. described a significant difference between males and females in the combined effect of multiple treatments on the lesion size, including an IL-1 receptor antagonist (IL-1ra). For IL-1ra a significantly smaller lesion size was described for both males and females. However, the authors did not formally assess the difference between sexes for this specific treatment separately (Elhage et al., 1998). The second study, by Traughber et al. mentioned that the lesion size was ~50 % smaller upon treatment in both male and female mice, but no formal assessment of the difference between males and females was provided (Traughber et al., 2023). The forest plot with these two studies is shown in Fig. 6.

The second scenario we envisioned were subgroup analyses regarding sex. The two studies investigating both males and females separately (Elhage et al., 1998; Traughber et al., 2023) were included in the subgroup analysis. Only the three groups "IL-1β inhibition indirect", "IL-18 inhibition indirect" and "inhibition total" had male subgroups that were large enough for formal subgroup analyses (Supplementary Table 8). For males the plaque size was significantly smaller upon inhibitory treatment, indicated by the negative SMD ± $CI_{95\ \%}$, which was similar to the overall effect (Table 3). The number of studies investigating IL-6/IL-6R interventions was too low for subgroup analyses.

In the forest plots in Fig. 7, the Hedge's g values and confidence intervals are displayed, which show that the inhibitory intervention studies generally show reduced lesion size upon treatment, indicated by the negative Hedge's g values (those below 0). Although the number of studies in the subgroup of females was too low to reliably interpret the subgroup effect, visual inspection of the forest plots suggested no difference in effect size between females and males, as the confidence intervals of the studies conducted in females completely overlap with the studies conducted in males (Fig. 7A and B).

3.5. *PB analysis*

The risk of potential publication bias could only be assessed for indirect inhibition of IL-1β (n = 16) and IL-18 (n = 16) and for inhibition total (n = 39). There was no evidence for publication bias according to Egger's regression test (indirect inhibition of IL-1β: p = 0.57, indirect inhibition of IL-18: p = 0.57 and total inhibition: p = 0.64).

4. Discussion

In this systematic review, we aimed to assess experimental animal sex in atherosclerosis studies with (anti-)inflammatory therapeutics and determine whether the sex of the animal is an important variable to be included in such *in vivo* atherosclerosis studies. To do so, we specifically focused on literature investigating the modulation of IL-1, IL-18 and IL-6 cytokine signaling in atherosclerotic animal models with atherosclerotic plaque size as main read-out. These inflammatory pathways were selected as they are of interest for clinical development to reduce the inflammatory cardiovascular risk upon lipid-lowering therapy. We here show, by including papers up to the end of 2023, that inhibition of these cytokine signaling pathways collectively reduced aortic plaque development in atherosclerotic mice. Subgroup analyses for the combined inhibition data showed a similar result in male mice. For female mice, no formal analyses could be conducted due to limited number of studies, however, visual inspection of the forest plot suggests no significant differences in effect between males and females.

In this systematic review, atherosclerotic plaque size was initially analyzed per cytokine or receptor, and in a second phase, the inhibitory

Table 1
Overall direction of effect on plaque size upon modulation of IL-1, IL-18, IL-6 or their receptors. Meta-analyses results for every target group, including the data of inhibition of all targets (inhibition total) or the data of stimulation of all targets (stimulation total). Number (N) of studies, N of comparisons, overall effect on plaque size (standardized mean difference (SMD) ± 95 % confidence interval ($CI_{95\ \%}$)) and I^2 are presented per group.

Cytokine signaling target	N studies	N comparisons	Overall effect (SMD ± $CI_{95\ \%}$)	I^2
IL-1α inhibition direct	3	4	−1.4 [−2.4 to −0.4]	80
IL-1α inhibition indirect	0	0	–	–
IL-1α stimulation direct	0	0	–	–
IL-1α stimulation indirect	0	0	–	–
IL-1β inhibition direct	7	12	−0.5 [−0.9 to −0.1]	51
IL-1β inhibition indirect	16	24	−2.2 [−2.7 to −1.6]	77
IL-1β stimulation direct	1	1	5.0 [2.8 to 7.3]	0
IL-1β stimulation indirect	2	3	2.0 [−0.1 to 4.1]	91
IL-1R inhibition direct	8	13	−1.0 [−1.6 to −0.5]	71
IL-1R inhibition indirect	1	1	−1.4 [−2.5 to −0.2]	0
IL-1R stimulation direct	0	0	–	–
IL-1R stimulation indirect	0	0	–	–
IL-18 inhibition direct	1	1	−0.8 [−1.5 to −0.1]	0
IL-18 inhibition indirect	16	24	−2.2 [−2.7 to −1.6]	77
IL-18 stimulation direct	4	6	0.9 [0.2 to 1.6]	68
IL-18 stimulation indirect	1	2	0.7 [0.0 to 1.4]	27
IL-6 inhibition direct	1	3	−2.0 [−3.2 to −0.7]	68
IL-6 inhibition indirect	3	3	−4.5 [−6.4 to −2.7]	52
IL-6 stimulation direct	1	1	−2.5 [−4.0 to −1.0]	0
IL-6 stimulation indirect	0	0	–	–
IL-6R inhibition direct	1	1	0.4 [−0.4 to 1.1]	0
IL-6R inhibition indirect	0	0	–	–
IL-6R stimulation direct	0	0	–	–
IL-6R stimulation indirect	0	0	–	–
Inhibition total	39	62	−1.5 [−1.8 to −1.2]	78
Stimulation total	8	11	1.2 [0.3 to 2.1]	86

interventions, and respectively the stimulating strategies were combined. In general, we show that inhibition of the pro-inflammatory cytokines significantly decreased plaque size, and stimulation significantly increased plaque size. An exception was a study with direct stimulation of IL-6, which led to a significantly smaller plaque size. This outcome could be explained by the controversial role of IL-6 in atherosclerosis (Reiss et al., 2017).

Our results confirm the importance of IL-1, IL-18 and IL-6 signaling in atherosclerosis and support the development of such anti-inflammatory therapies for human application. Strengthening this conclusion is the relevance of these studies to human disease, as in the CANTOS trial the recurrence of cardiovascular events was reduced upon anti-IL-1β treatment (Ridker et al., 2017), and in the RESCUE trial treatment of high cardiovascular risk patients with an anti- IL-6 antibody lowered, hsCRP (Ridker et al., 2021). Although plaque measurements were not an endpoint in these clinical trials, plaque burden is shown to be a predictor of cardiovascular events in patients (Erlinge et al., 2021; McPherson et al., 2012; Stone et al., 2011; Vergallo et al., 2025). Therefore, the outcome parameter in this systematic review, plaque size, is an important aspect in the translation to human disease, but it is important to note that plaque composition aids in defining whether a plaque is high-risk. In future systematic review studies on inflammatory therapies in animal models of atherosclerosis, plaque composition would be a useful addition to the outcome parameters.

The aim of this study was to explore potential sex differences in therapeutic efficacy of targeting IL-1, IL-18 and IL-6 cytokine pathways in preclinical atherosclerosis models, which would provide knowledge on which sex to include in such studies or whether animal sex can be excluded as a variable. Surprisingly, studies using female mice were very limited as evident from our analysis, and mainly male mice were used for the included studies, at least in papers up to the end of 2023. The observations from our subgroup analyses show that therapeutic efficacy is highly significant in male mice, rendering the male mouse a valid model for such studies. From the limited number of studies with female mice, therapeutic efficacy seemed similar as compared to male mice, however due to the lack of power we cannot formally conclude this. In general, animal atherosclerosis studies are mostly performed in either male or female animals. Studies conducted in both sexes rarely contain a statistical test to determine whether sex is an independent variable (Man et al., 2020). To enable formal tests on sex differences, we recommend scientists to include both males and females in their studies, and assess the difference between the sexes. In our review, we only found two studies that used both male and females separately (Elhage et al., 1998; Traughber et al., 2023), but neither formally assessed the difference in effect on lesion size between males and females for our treatment of interest. Previously, male mice were shown to have more inflamed, but smaller lesions compared to females (Man et al., 2020). These differences strengthen our recommendation to use both sexes in mice studies to enable the establishment of sex effects in anti-inflammatory studies, in line with the ARRIVE guidelines as well as the recommendations from the American Heart Association (Daugherty et al., 2017). These differences in inflammation, also shows the importance of inclusion of additional plaque components as outcome parameters in future systematic reviews.

This is the first systematic review in the field investigating animal sex in studies assessing the efficacy of preclinical anti-inflammatory treatments. Our main conclusions are based on studies in which 55 % of the mice were 16–23 weeks old and 29 % 24–36 weeks. Mice are defined mature adult in the age range from 3 to 6 months, middle aged from 10 to 15 months, old from 18 to 24 months and very old over 24 months. Thus, our conclusions are based on relatively young mice. Man et al. summarized that in general, female *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice have larger plaques compared to male mice this comprises for *Apoe*$^{-/-}$ in the aortic root through 6 months age, in the whole aorta of 4–12 months old on a normal chow diet, and for *Ldlr*$^{-/-}$ in the aortic root and whole aorta through 6 months of age (Man et al., 2020). However, there are some exceptions (Man et al., 2020). A number of studies show a similar plaque size for 1–15 months male and female *Apoe*$^{-/-}$ mice on a normal chow

Table 2
Sensitivity analysis for IL-1β and IL-18. The NLRP3 inflammasome and caspase-1 comparisons described in the right part of the table are included in the meta-analyses, but not in the sensitivity analyses of the IL-1β and IL-18 groups. Number (N) of studies (stu), N of comparisons (com), overall effect on plaque size (standardized mean difference (SMD) ± 95 % confidence interval ($CI_{95\ \%}$)) and I^2 are presented for the analyses.

Cytokine signaling target	Meta-analysis				Sensitivity analysis				NLRP3 inflammasome and caspase-1 comparisons included in IL-1β and IL-18 meta-analyses		
	N stu	N com	Overall effect (SMD ± $CI_{95\ \%}$)	I^2	N stu	N com	Overall effect (SMD ± $CI_{95\ \%}$)	I^2	Cytokine signaling target	N stu	N com
IL-1β inhibition direct	7	12	−0,5 [−0,9 to −0,1]	51	7	12	−0,5 [−0,9 to −0,1]	51	NLRP3 inflammasome inhibition direct	0	0
IL-1β inhibition indirect	16	24	−2,2 [−2,7 to −1,6]	77	0	0	–	–	NLRP3 inflammasome inhibition indirect	14	19
IL-1β stimulation direct	1	1	5,0 [2,8 to 7,3]	0	1	1	5,0 [2,8 to 7,3]	0	NLRP3 inflammasome stimulation direct	0	0
IL-1β stimulation indirect	2	3	2,0 [−0,1 to 4,1]	91	1	1	5,3 [3,3 to 7,2]	0	NLRP3 inflammasome stimulation indirect	1	2
IL-18 inhibition direct	1	1	−0,8 [−1,5 to −0,1]	0	1	1	−0,8 [−1,5 to −0,1]	0	Caspase-1 inhibition direct	0	0
IL-18 inhibition indirect	16	24	−2,2 [−2,7 to −1,6]	77	0	0	–	–	Caspase-1 inhibition indirect	2	5
IL-18 stimulation direct	4	6	0,9 [0,2 to 1,6]	68	4	6	0,9 [0,2 to 1,6]	68	Caspase-1 stimulation direct	0	0
IL-18 stimulation indirect	1	2	0,7 [0,0 to 1,4]	27	0	0	–	–	Caspase-1 stimulation indirect	0	0

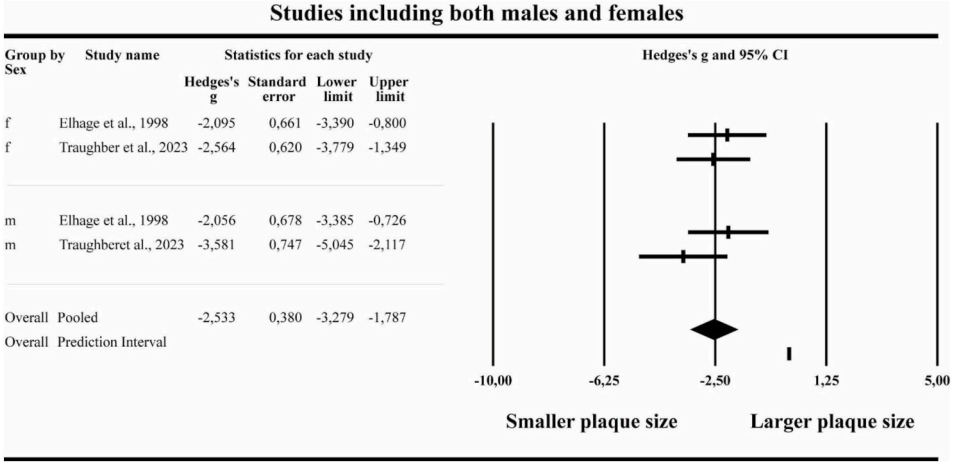

Fig. 6. Forrest plot for plaque size of studies that investigated both males and females. The numbers are the Hedges's g, standard error and the lower and upper limit of the 95 % confidence interval (=$CI_{95\ \%}$). The diamond indicates the overall pooled effect (Elhage et al., 1998; Traughber et al., 2023).

Table 3
Male subgroup analyses for plaque size upon cytokine signaling inhibition. Number (N) of studies and comparisons, overall effect on plaque size (standardized mean difference (SMD) ±95 % confidence interval ($CI_{95\ \%}$)) per sex, I^2 are presented.

Cytokines signaling target	Sex	N studies	N comparisons	Effect (SMD ± $CI_{95\ \%}$)	I^2
IL-1β/IL-18 inhibition indirect	Overall	16	22	−2,2 [−2,7 to −1,6]	77
	Females	2	3	Not calculated	
	Males	12	15	−2,3 [−3,0 to −1,6]	74
Inhibition total	Overall	39	62	−1,5 [−1,8 to −1,2]	78
	Females	7	10	Not calculated	
	Males	28	42	−1,6 [−1,9 to −1,2]	79

diet in the whole aorta (Champagne et al., 2009; Jeon et al., 2010; Liu et al., 2016; Maier et al., 2017; Marek et al., 2017; Matsumoto et al., 2016) and some studies show that 3–8 months male *Apoe*$^{-/-}$ mice have larger lesions in the whole aorta than females on a normal chow diet (Liu et al., 2016; Zhang et al., 2018). *Apoe*$^{-/-}$ mice, up to 22 weeks of age and on an atherogenic diet have similar plaques in male and female whole aortas. However, in some studies with a long duration of an atherogenic diet male *Apoe*$^{-/-}$ mice of 6 or 12 months old had larger lesions than females (Chiba et al., 2011; Tangirala et al., 1995). For studies using *Ldlr*$^{-/-}$ mice, there are some exceptions as well. One study using 6 months old male mice showed larger lesions in the descending aorta compared to female mice after 5 months of an atherogenic diet (Parks et al., 2006). In a similar study, one-year-old males had larger lesions compared to females in the whole aorta after 6 months of an atherogenic diet (Tangirala et al., 1995). Smit et al. showed that the lesion size in the aortic root of 22 months old *Ldlr*$^{-/-}$ mice was similar in males and females (Smit et al., 2024). Overall, the age of the mice should be taken into account when considering the exclusion of sex as a variable.

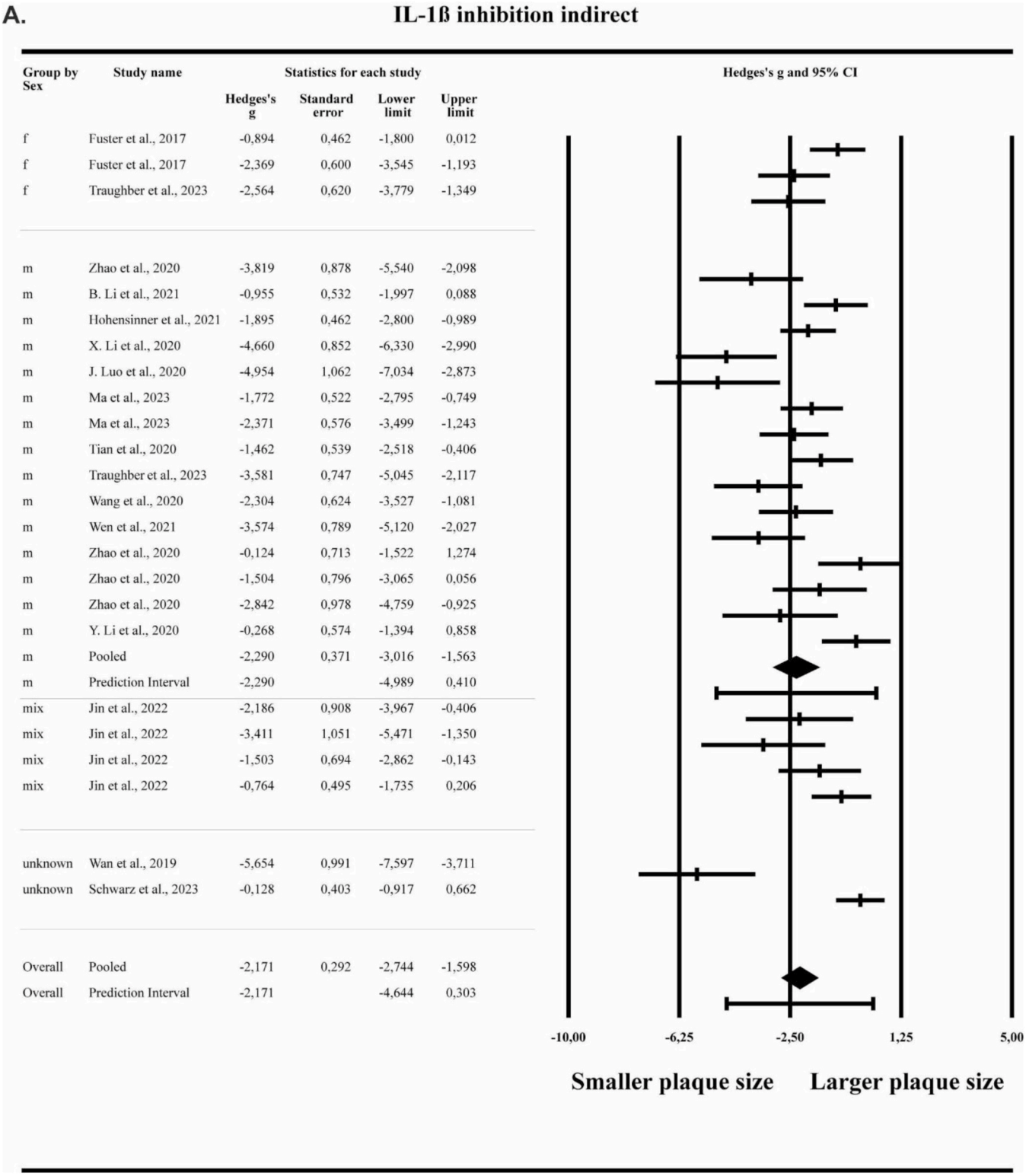

Fig. 7. Forrest plot for plaque size for the groups with enough studies for a male subgroup analysis. A. For the IL-1β or IL-18 signaling inhibition group. B. For the total cytokine signaling inhibition group. The numbers are the Hedges's g, standard error and the lower and upper limit of the 95 % confidence interval (= $CI_{95\ \%}$). The diamonds indicate the pooled effect of the male subgroup and overall.

To account for the inclusion of NLRP3 inflammasome and caspase-1 targeting in both IL-1β and IL-18 groups for meta-analyses, we performed sensitivity analyses. These indicated that our results were robust and our overall conclusions were not affected. In addition, analyses of between-study heterogeneity revealed moderate to severe levels. Heterogeneity is expected in animal research due to the often-exploratory approach, meaning some of it is intentionally induced. To address this, we applied a random effects model, and examined potential causes

B. **Inhibition total**

Group by Sex	Study name	Hedges's g	Standard error	Lower limit	Upper limit
f	Christersdottir et al., 2019	-1,351	0,562	-2,452	-0,250
f	Dragoljevic et al., 2020	-0,713	0,504	-1,701	0,275
f	Elhage et al., 1998	-2,095	0,661	-3,390	-0,800
f	Fidler et al., 2021	-0,179	0,474	-1,108	0,750
f	Fidler et al., 2021	-0,181	0,478	-1,117	0,755
f	Fidler et al., 2021	0,170	0,282	-0,382	0,722
f	Fuster et al., 2017	-0,894	0,462	-1,800	0,012
f	Fuster et al., 2017	-2,369	0,600	-3,545	-1,193
f	Traughber et al., 2023	-2,564	0,620	-3,779	-1,349
f	Yalcinkaya et al., 2023	-0,470	0,361	-1,178	0,238
m	Bhaskar et al., 2011	-1,573	0,686	-2,917	-0,229
m	Bhaskar et al., 2011	-0,906	0,638	-2,155	0,344
m	Bhaskar et al., 2011	-1,207	0,679	-2,538	0,124
m	Chen et al., 2012	-2,324	0,564	-3,430	-1,218
m	Denes et al., 2012	-0,595	0,405	-1,388	0,199
m	Denes et al., 2012	-0,565	0,498	-1,541	0,411
m	Elhage et al., 1998	-2,056	0,678	-3,385	-0,726
m	Gomez et al., 2018	0,086	0,533	-0,960	1,131
m	Hohensinner et al., 2021	-1,895	0,462	-2,800	-0,989
m	Karnewar et al., 2024	2,328	0,773	0,813	3,842
m	Ku et al., 2022	0,211	0,614	-0,992	1,414
m	Ku et al., 2022	-1,771	0,704	-3,150	-0,391
m	Ku et al., 2022	-1,103	0,649	-2,376	0,170
m	B. Li et al., 2021	-0,955	0,532	-1,997	0,088
m	X. Li et al., 2020	-4,660	0,852	-6,330	-2,990
m	Y. Li et al., 2020	-0,268	0,574	-1,394	0,858
m	M. Liu et al., 2020	-0,836	0,448	-1,715	0,042
m	W. Liu et al., 2023	0,358	0,364	-0,355	1,072
m	J. Luo et al., 2020	-4,954	1,062	-7,034	-2,873
m	P. Luo et al., 2020	-4,567	1,297	-7,109	-2,025
m	P. Luo et al., 2021	-6,634	1,455	-9,486	-3,782
m	Ma et al., 2023	-1,772	0,522	-2,795	-0,749
m	Ma et al., 2023	-2,371	0,576	-3,499	-1,243
m	Mallat et al., 2001	-0,803	0,358	-1,504	-0,103
m	Meng et al., 2016	-2,677	0,633	-3,918	-1,436
m	Schuett et al., 2012	-0,916	0,572	-2,037	0,205
m	Schuett et al., 2012	-3,310	0,782	-4,842	-1,778
m	Schuett et al., 2012	-1,912	0,553	-2,995	-0,828
m	Tian et al., 2020	-1,462	0,539	-2,518	-0,406
m	Tissot et al., 2013	-1,768	0,471	-2,691	-0,845
m	Traughber et al., 2023	-3,581	0,747	-5,045	-2,117
m	Vromman et al., 2019	-1,269	0,492	-2,232	-0,305
m	Vromman et al., 2019	-0,023	0,449	-0,903	0,857
m	Vromman et al., 2019	-0,230	0,419	-1,052	0,592
m	Vromman et al., 2019	-0,724	0,431	-1,569	0,121
m	Wang et al., 2020	-2,304	0,624	-3,527	-1,081
m	Wen et al., 2021	-3,574	0,789	-5,120	-2,027
m	Xu et al., 2023	-4,067	1,343	-6,700	-1,434
m	Zhao et al., 2020	-3,819	0,878	-5,540	-2,098
m	Zhao et al., 2020	-0,124	0,713	-1,522	1,274
m	Zhao et al., 2020	-1,504	0,796	-3,065	0,056
m	Zhao et al., 2020	-2,842	0,978	-4,759	-0,925
m	Pooled	-1,559	0,193	-1,938	-1,180
m	Prediction Interval	-1,559		-3,726	0,609
mix	Hettwer et al., 2022	-0,836	0,414	-1,646	-0,025
mix	Hettwer et al., 2022	-0,973	0,427	-1,810	-0,136
mix	Jin et al., 2022	-2,186	0,908	-3,967	-0,406
mix	Jin et al., 2022	-3,411	1,051	-5,471	-1,350
mix	Jin et al., 2022	-1,503	0,694	-2,862	-0,143
mix	Jin et al., 2022	-0,764	0,495	-1,735	0,206
unknown	Schwarz et al., 2023	-0,128	0,403	-0,917	0,662
unknown	Takeda et al., 2006	-3,352	0,758	-4,838	-1,866
unknown	Wan et al., 2019	-5,654	0,991	-7,597	-3,711
unknown	Wu et al., 2022	-1,372	0,583	-2,514	-0,229
Overall	Pooled	-1,491	0,158	-1,800	-1,182
Overall	Prediction Interval	-1,491		-3,610	0,627

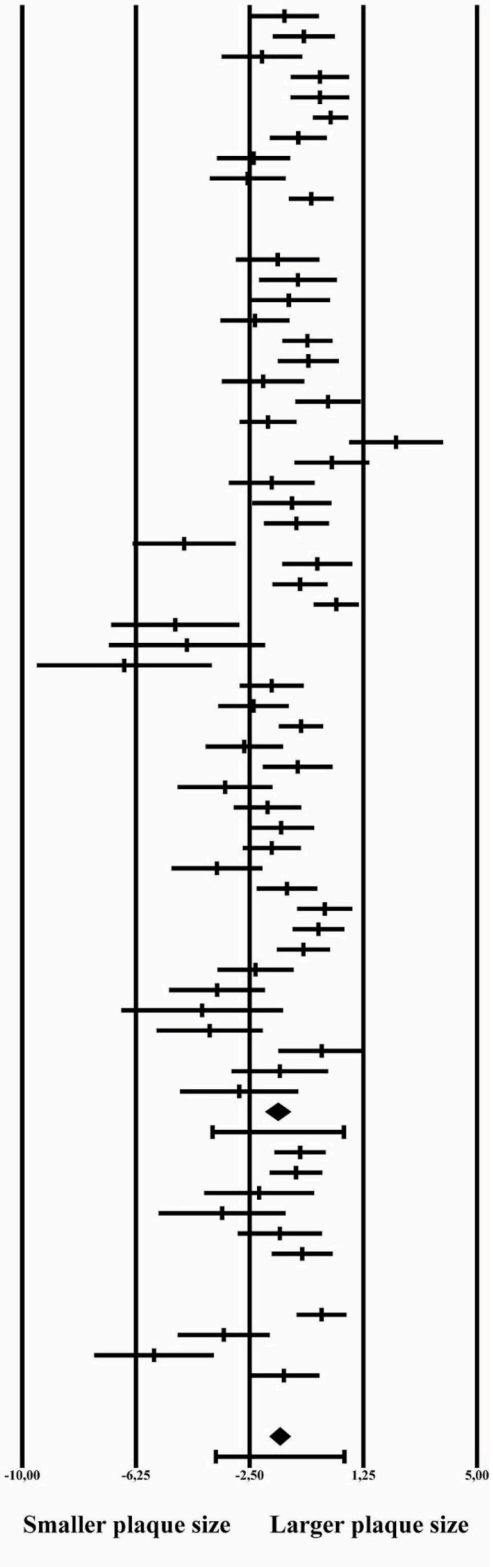

Fig. 7. (*continued*).

of heterogeneity through subgroup analyses regarding sex. Unfortunately, the number of studies using females was too low to formally assess. Furthermore, in this systematic review only studies using mice were identified. Whether the effects are similar in other species remains unclear, however the number of atherosclerosis studies in other animal models is very limited. Overall, most of the animal studies that we included failed to report a number of important study details, hampering risk of bias analyses. While this is common in the field, the lack of reporting on important methodological details suggests a certain degree of neglect of methods designed to reduce bias, potentially leading to skewed results and severely hindering the ability to draw reliable conclusions from the included animal studies.

5. Conclusion

All together, we show that inhibition of the cytokine signaling pathways of IL-1, IL-6 and IL-18 collectively reduced aortic plaque development in atherosclerotic mice, which was predominantly studied in male mice. Our analyses into sex differences suggest no difference between males and females, although we could not formally test this due to the limited number of studies in the female subgroup. More studies using female animals in this research area are thus needed.

CRediT authorship contribution statement

Lucie Delfos: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Cody Huis:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Nadia Imani:** Investigation, Writing – review & editing. **Jeanine E. Roeters van Lennep:** Conceptualization. **Carlijn R. Hooijmans:** Conceptualization, Formal analysis, Investigation, Methodology, Resources, Writing – review & editing. **Ilze Bot:** Conceptualization, Funding acquisition, Investigation, Methodology, Supervision, Writing – review & editing.

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Declaration of competing interest

The author is an Editorial Board Member for *European Journal of Pharmacology* and was not involved in the editorial review or the decision to publish this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2025.178336.

Data availability

We have used publicly available data for this systematic review. We have included the data used in the manuscript.

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