A short time window to profit from protection of blood-induced cartilage damage by IL-4 plus IL-10

Monique E. R. van Meegeren1,2, Goris Roosendaal2, Karin van Veghel1, Simon C. Mastbergen1 and Floris P. J. G. Lafeber1

Abstract

Objective. IL-4 plus IL-10 prevents blood-induced cartilage damage. The aim of the present study was to evaluate whether cartilage damage can still be averted by addition of IL-4 plus IL-10 when added after the onset of a bleed and whether aspiration of blood prior to addition of IL-4 plus IL-10 is of additive protective value.

Methods. Healthy canine hip and human shoulder cartilage was exposed to whole blood for 4 days. IL-4 plus IL-10 was administered directly or after a delay of several hours up to 2 days. Furthermore, blood was aspirated after 1 or 2 days and subsequently IL-4 plus IL-10 was added. IL-1β concentration and cartilage matrix proteoglycan turnover were determined.

Results. Exposure of canine and human cartilage to blood decreased the proteoglycan synthesis rate and content and increased proteoglycan release. IL-4 plus IL-10 only prevented blood-induced damage of canine cartilage when added directly, not after 4 h or later. For human cartilage, IL-4 plus IL-10 limited blood-induced damage as well as IL-1β production when administered within 4–8 h after the onset of a bleed, but not thereafter. Aspiration of blood within 24 h fully prevented cartilage damage. Subsequent addition of IL-4 plus IL-10 was not of additive value.

Conclusion. For humans, there is a short time window after onset of a joint bleed in which IL-4 plus IL-10 can limit blood-induced cartilage damage. Furthermore, aspiration of a joint to shorten blood exposure fully prevents cartilage damage. Both options can be considered in the treatment of a joint haemorrhage.

Key words: haemarthrosis, trauma, joint surgery, haemophilia, IL-4, IL-10, aspiration.

Introduction

A joint bleed occurring after major joint trauma or surgery or a spontaneous joint bleed in a haemophilia patient causes damage to the articular cartilage [1, 2]. Previous in vitro research has shown that a single exposure of cartilage to blood leads to long-lasting, irreversible damage [3]. This is caused by induction of chondrocyte apoptosis through formation of hydroxyl radicals [4]. IL-1β produced by activated monocytes/macrophages increases the production of hydrogen peroxide by chondrocytes. Hydroxyl radicals are subsequently formed when hydrogen peroxide reacts with erythrocyte-derived catalytic iron in the vicinity of chondrocytes [5].

In addition to these direct effects on cartilage, there is synovial tissue inflammation. Natural evacuation of blood from the joint cavity leads to deposition of iron (haemosiderin) in the synovial tissue. This results in proliferation and hypertrophy of the synovium, fibrosis and neovascularization [6]. Infiltration of the synovial tissue with lymphocytes causes a more chronic inflammatory reaction, contributing to cartilage damage [7].

Surprisingly there is no consensus on aspiration (arthrocentesis) of a joint after a joint bleed, neither in the case of haemophilia nor after major joint trauma [8, 9], even though several studies have proved the benefits of aspiration [10, 11]. In addition to the prevention of joint bleeds during surgery and quick aspiration of a joint after a bleed, the devastating effects of blood in a joint may be averted by treatment.
Modulatory/anti-inflammatory cytokines like IL-4 and IL-10 can protect cartilage from damage in arthritis. IL-4 inhibits production of IL-1β and TNF-α of stimulated SF mononuclear cells (MNC) [12]. In animal models of inflammatory arthritis, IL-4 inhibits cartilage and bone degradation as well as inflammation [13–16]. IL-10 inhibits production of IL-1β and TNF-α by activated macrophages in vitro [17] and production of metalloproteinase by MNC and stimulates production of their inhibitor (TIMP-1) [18]. Because IL-4 and IL-10 use different signalling pathways, they are able to exert different, but potentially additive effects. It has been reported that IL-10 acts synergistically with IL-4 in suppressing macrophage cytotoxic activity [19] and production of IL-1 receptor antagonist by human neutrophils [20]. Additionally, the combination of IL-4 and IL-10 has an additive effect on the inhibition of IL-1β and TNF-α production by antigen-stimulated MNC from the SF and peripheral blood from patients with RA [21].

It has also been shown that IL-10 protects cartilage from the damaging effects of blood exposure in terms of cartilage matrix turnover [22]. Moreover, in vitro, IL-10 beneficially affects cartilage and synovial tissue from patients with haemophilic arthropathy [22]. We recently showed that a combination of IL-4 plus IL-10 protects cartilage from blood-induced damage when administered directly upon blood exposure to a greater extent than IL-10 alone [23]. However, this is not of value for clinical practice because a patient can never be treated at the exact same time as the joint bleed starts. Therefore, in this study, the clinical relevance of IL-4 plus IL-10 administration is evaluated by investigating whether IL-4 plus IL-10 is still able to protect cartilage from blood-induced damage when added after the onset of a bleed and whether addition of IL-4 and IL-10 early in time is of additive value to aspiration of the blood.

Materials and methods

Canine in vitro experiments

Healthy full-thickness beagle dog articular cartilage was obtained post-mortem from hips (n = 12 females, mean age 2.1 ± 0.1 years) within 3 h after death of the animal. These animals were used for other studies and cartilage of hip joints could be obtained as surplus tissue. The Utrecht University Animal Experiments Committee gave ethics approval for the animal studies. Explants were cultured individually in a 96-well round-bottomed microtitre plate containing 200 μl of medium per well in a tissue incubator (5% CO2 in air, pH 7.4, 37°C). Culture medium consisted of DMEM (Invitrogen) supplemented with glutamine (2 mM), penicillin (100 IU/ml), streptomycin sulphate (100 μg/ml; all from PAA Laboratories), ascorbic acid (85 μM; Sigma) and 10% heat-inactivated pooled beagle dog serum.

Cartilage of the 12 dogs was exposed to 20% v/v whole blood (by adding 40 μl of blood to 160 μl of culture medium) for 4 days, assuming the blood is cleared relatively quickly from the canine joint, resulting in low blood levels within a 4-day time span [24]. A percentage of 20% v/v was chosen as compared with previous human in vitro studies [25], since canine cartilage is thinner compared with human cartilage and therefore more sensitive to blood-induced cartilage damage. Fresh blood was drawn from healthy dogs (in total n = 3, mean age 2.3 ± 0.3 years) in a heparinized vacutainer tube (catalogue no. 367820, Becton Dickinson). A combination of 30 ng/ml recombinant canine IL-4 and IL-10 (R&D Systems) was added directly after the addition of blood, as well as 4 and 8 h after the start of blood exposure. Experiments demonstrated that this concentration causes optimal protection against blood-induced damage [23] and is a concentration feasible to administer intra-articularly in vivo [26]. After 4 days exposure to blood without and with IL-4 plus IL-10, cartilage explants were washed twice under culture conditions for 45 min. Subsequently cartilage was cultured for an additional 12 days without additions, during which culture medium was refreshed every 4 days.

Human in vitro experiments

To evaluate whether the effects observed with canine cartilage were also obtained with human cartilage tissue, and to extend different variables, a comparable set of experiments was performed with human cartilage tissue. Healthy human full-thickness articular cartilage tissue was obtained post-mortem from humeral heads within 24 h after death of the donor. The Utrecht University Medical Research Ethics Committees for human medical research approved collection of healthy cartilage with permission of the patient’s family, since collection of material was post-mortem. The donors (n = 16, mean age 65 ± 4 years, 10 males and 6 females) had no known history of joint disorders. Cartilage was cut aseptically and the underlying bone was excluded. Slices were kept in PBS (pH 7.4). Within 1 h after dissection, slices were cut in small cubes and weighed aseptically (range 5–15 mg, accuracy ± 0.1 mg).

Cartilage was cultured in the same culture medium as canine cartilage explants, except that 10% heat-inactivated pooled human AB+ serum (Gemini Bioproducts) was used instead of canine serum. For each experiment, fresh blood was drawn from healthy human donors (n = 16, mean age 29 ± 2 years, 5 males and 11 females) in a vacutainer tube (catalogue no. 367880, Becton Dickinson). Explants were exposed to 50% v/v whole blood for 4 days, mimicking the concentration and duration observed during the natural evacuation of blood from a human joint [25]. After blood exposure, cartilage explants were washed twice under culture conditions for 45 min and cultured for an additional 12 days. Culture medium was refreshed every 4 days without further additions.

In a first set of experiments, a combination of 30 ng/ml recombinant human IL-4 and IL-10 (both Sigma) was added directly after addition of blood or 2, 4, 8, 24 or 48 h after the onset of blood exposure. In addition to proteoglycan turnover, IL-1β was determined in these experiments as a measure of proinflammatory and cartilage
destructive cytokines and a key player in the pathway leading to chondrocyte apoptosis [5].

In a second set of experiments, the cartilage explants were put in fresh culture medium after 1 or 2 days to remove most of the blood from the cartilage (without thorough rinsing), mimicking aspiration of a joint. Subsequently 30 ng/ml IL-4 and IL-10 was added. At day 4, all the remaining blood and cytokines were removed, mimicking the human in vivo conditions as closely as possible [25], and cultures proceeded for 12 days follow-up.

Determination of proteoglycan turnover
The proteoglycan synthesis rate, the release of proteoglycans from the cartilage matrix into the culture medium and the proteoglycan content of the cartilage explant were measured as described earlier [23].

IL-1β ELISA
IL-1β levels were determined after 0, 2, 4, 8 and 24 h in blood (50% v/v) cultures with or without cartilage. Subsequently, the effect of IL-4 plus IL-10 addition after 2, 4, 8, 24 and 48 h was determined in blood cultures at day 4. Culture media were centrifuged at 1500 g for 10 min and stored at −80°C until analysis. A commercially available ELISA Cytoset (Biosource) was used and the analysis was performed according to the manufacturer’s instructions.

Calculations and statistical analyses
Each experiment was performed with cartilage obtained from a single donor. Because joint cartilage from different locations within a joint varies in composition and cellular activity, the mean value of 10 cartilage explants per parameter per donor, obtained randomly and handled individually, was taken as a representative value for the cartilage of that donor (10 replicates for each experiment). The n-values in this study indicate the number of cartilage donors (experiments). Results were depicted as median values ± interquartile range (IQR). The data were analysed using a non-parametric test for related samples (Wilcoxon signed-rank test) with SPSS 15.0 software. Differences were considered statistically significant at \( P < 0.05 \).

Results
Effects of IL-4 plus IL-10 on blood-induced canine cartilage damage
The proteoglycan synthesis rate of canine cartilage cultured in vitro decreased on average by 74% after exposure to 20% v/v blood (Fig. 1A; \( P = 0.002 \)). Administration of IL-4 plus IL-10 concomitantly with the blood resulted in a significant recovery of the proteoglycan synthesis rate (\( P = 0.002 \)). The synthesis rate almost normalized, no longer being statistically significantly different from controls (\( P = 0.071 \)). However, when the combination of IL-4 plus IL-10 was added 4 and 8 h after the onset of blood exposure, the proteoglycan synthesis rate remained decreased on average by 75% and 70%, respectively, when compared with controls (both \( P = 0.002 \)). These values were not different from blood exposure alone (\( P = 0.455 \) and \( P = 0.346 \), respectively).

Release of proteoglycans from the cartilage matrix was increased by 40% after blood exposure (Fig. 1B; \( P = 0.005 \)). Concomitant addition of IL-4 plus IL-10 counteracted the harmful effect of blood (\( P = 0.037 \)); proteoglycan release was now enhanced by only 21% and was not statistically significantly different from control conditions (\( P = 0.139 \)). The addition of IL-4 plus IL-10 after 4 or 8 h could not prevent an increase in proteoglycan release due to blood exposure (\( P = 0.013 \) and \( P = 0.009 \), respectively, when compared with controls). These values were not different from blood exposure (\( P = 0.285 \) and \( P = 0.114 \), respectively).

The impaired proteoglycan synthesis rate and the increased release after exposure to blood resulted in a decrease of proteoglycan content by 45% (Fig. 1C; \( P = 0.002 \)) 12 days after the 4-day blood exposure. The addition of IL-4 plus IL-10 together with blood exposure prevented this decrease (\( P = 0.005 \)); on average, only 16% inhibition remained, although still statistically significantly different from control conditions (\( P = 0.041 \)). When IL-4 plus IL-10 was added 4 or 8 h after the start of blood exposure, proteoglycan content remained decreased by 27% and 46%, respectively, when compared with controls (\( P = 0.003 \) and \( P = 0.002 \), respectively) and was not different from blood exposure alone (\( P = 0.347 \) and \( P = 0.456 \), respectively).

Effects of IL-4 plus IL-10 on blood-induced human cartilage damage
Human cartilage exposed to 50% v/v whole blood showed an average decrease in the proteoglycan synthesis rate of 76% (Fig. 2A; \( P < 0.001 \)). The addition of IL-4 and IL-10 immediately after addition of blood caused a significant recovery of the proteoglycan synthesis rate (\( P < 0.001 \) compared with blood), matching control values. Without addition of blood, IL-4 plus IL-10 does not exert any effect [23]. When the combination of IL-4 plus IL-10 was administered 2 or 4 h after the start of blood exposure, the proteoglycan synthesis rate still recovered significantly (\( P = 0.028 \) and \( P = 0.018 \), respectively, compared with blood). When added at later time points, IL-4 plus IL-10 had no effect on the inhibited decrease.

Proteoglycan release from the human cartilage matrix was increased by 36% after 4 days of blood exposure (Fig. 2B; \( P = 0.004 \)). The addition of blood to cartilage immediately followed by the addition of IL-4 plus IL-10 prevented the increased release of proteoglycans (\( P = 0.001 \) compared with blood). The release of proteoglycans was even slightly lower than control values (reduction of 18%; \( P = 0.008 \)). Beneficial recovery was also observed in case IL-4 plus IL-10 was added within the first 24 h after the start of blood exposure (for 2, 4, 8 and 24 h, \( P = 0.016 \), \( P = 0.043 \), \( P = 0.018 \) and \( P = 0.017 \), respectively). When IL-4 plus IL-10 was added 48 h after the onset of blood exposure, it was unable to change the enhanced release induced by blood exposure.
Decreased proteoglycan synthesis rate and enhanced proteoglycan release due to blood exposure of human cartilage explants eventually led to a decrease of total proteoglycan content by 15% (Fig. 2C; \( P = 0.005 \)). Treatment with IL-4 plus IL-10 immediately after addition of blood prevented a blood-induced decrease in proteoglycan content (\( P = 0.005 \) compared with blood); it was only decreased by 7% and was no longer statistically significantly different from control values. A delay in the addition of IL-4 plus IL-10 of 8 h or more was unable to recover the loss of proteoglycans. A decrease in proteoglycan content by 5-7% compared with controls was measured, not statistically significantly different from blood alone (\( P = 0.176, P = 0.400 \) and \( P = 0.233 \) for addition after 8, 12 and 24 h, respectively).

**Effects of IL-4 plus IL-10 on IL-1β production**

Fig. 3A demonstrates that after 24 h of culture, IL-1β protein levels were detectable and not different between 50% blood cultures with and without cartilage. After 4 days of culture, IL-1β concentration reached levels of up to 250 pg/ml (Fig. 3B; \( P = 0.028 \) compared with controls). Concomitant administration of IL-4 plus IL-10, as well as 2 or 4 h after onset, decreased IL-1β concentration to the levels of control values (all \( P = 0.028 \) compared with blood). This decrease in IL-1β was still observed, although less pronounced, when IL-4 plus IL-10 was administered 8 h after the start of the blood exposure (\( P = 0.028 \) compared with blood). When the delay in the addition of IL-4 plus IL-10 became longer (24 or 48 h), IL-1β levels were comparable to levels observed for blood alone (262 and 269 pg/ml, respectively) and were statistically significantly different from controls (\( P = 0.046 \) and \( P = 0.028 \), respectively).

**Effects of blood aspiration on cartilage damage**

In this set of experiments, 4 days exposure of human cartilage to 50% blood led to an average decrease of 70% in proteoglycan synthesis rate (Fig. 4A; \( P = 0.005 \)). When blood was removed 1 or 2 days after the start of exposure to cartilage, the proteoglycan synthesis rate was significantly less inhibited, by 17% and 44%, respectively (\( P = 0.046 \) and \( P = 0.028 \), compared with 4 days blood.
exposure). Nevertheless, the proteoglycan synthesis rate was still inhibited compared with controls (both $P = 0.028$). Surprisingly, administration of IL-4 and IL-10 directly after removal at day 1 or 2 did not have an additional beneficial effect in preventing the disturbance of cartilage matrix turnover compared with removal of blood alone ($P = 0.249$ and $P = 0.075$, respectively).

The release of proteoglycans from the cartilage was increased by 29% after 4 days of blood exposure (Fig. 4B; $P = 0.028$). When blood was removed from the cartilage 1 day after the start of culture, proteoglycan release was no longer statistically different from control values ($P = 0.028$ compared with 4 days exposure). Aspiration of blood from cartilage after 2 days could not prevent the enhanced release (27% increase compared with controls; $P = 0.075$). Addition of IL-4 and IL-10 directly after aspiration at day 1 or 2 was of no additional benefit ($P = 0.116$ and $P = 0.600$, respectively).

A 4-day blood exposure led to a decrease of total proteoglycan content of 15% (Fig. 4C; $P = 0.047$). When blood was removed from the cartilage after 1 day, proteoglycan content remained similar to the controls. This was statistically significantly different from 4 days blood exposure ($P = 0.028$). When blood was aspirated from cartilage after 2 days, proteoglycan content was still inhibited, at 7%, although no longer statistically significantly different from control cartilage ($P = 0.116$). Addition of IL-4 and IL-10 directly after removal of blood on day 1 or 2 did not improve the proteoglycan content when compared with aspiration alone ($P = 0.249$ and $P = 0.600$, respectively).

Discussion
This study demonstrates that IL-4 plus IL-10 can prevent blood-induced cartilage damage, but it needs to be administered directly or at least shortly after blood exposure to counteract the damaging effects of blood. Administration of IL-4 plus IL-10 to blood-exposed human cartilage within 4–8 h results in normalization of proteoglycan turnover, expectedly modulated by inhibition of blood-induced IL-1

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**Fig. 2 Effects of IL-4 plus IL-10 on human cartilage exposed to blood.**

Human articular cartilage was exposed to 50% v/v whole blood for 4 days. Human IL-4 plus IL-10 was added at a concentration of 30 ng/ml directly after blood exposure (0) and 2, 4, 8, 24, or 48 h after the start of blood exposure. Proteoglycan synthesis rate (A), release (B) and content (C) are depicted. Asterisks indicate a statistically significant difference from control values, whereas hash tags indicate a statistically significant difference from blood-exposed cartilage without IL-4 plus IL-10 addition ($P < 0.05$). Exact $P$-values are given in the text. Median values ± IQR of at least six individual cartilage donors (10 replicates each) and blood donors are shown.
Fig. 3 IL-1β production by blood cells and cartilage.

During 24 h, 50% blood was cultured in the presence or absence of a cartilage explant (A). IL-1β concentration of the supernatants was measured at the start of culture (0) and after 2, 4, 8 and 24 h. Median values of four individual blood cultures are shown. During a 4-day culture of 50% v/v blood, human IL-4 plus IL-10 was added in a concentration of 30 ng/ml directly at the start (0) and 2, 4, 8, 24 or 48 h after onset of culture (B). IL-1β concentration was measured at day 4. Asterisks indicate a statistically significant difference from control values, whereas hash tags indicate a statistically significant difference from blood-exposed cartilage without IL-4 plus IL-10 addition \( (P < 0.05) \). Exact \( P \)-values are given in the text. Median values ± IQR of six individual cartilage and blood donors are shown.

Fig. 4 Effects of the removal of blood combined with administration of IL-4 plus IL-10 on cartilage damage.

Human articular cartilage was exposed to 50% v/v whole blood for 4, 2 or 1 days. Subsequently, IL-4 and IL-10 was added in a concentration of 30 ng/ml. Proteoglycan synthesis rate (A), release (B) and content (C) are depicted. Asterisks indicate a statistically significant difference with control values, whereas hash tags indicate a statistically significant difference with cartilage exposed to blood for 4 days without the addition of IL-4 plus IL-10 \( (P < 0.05) \); median values of at least eight individual cartilage and blood donors ± IQR are shown. Exact \( P \)-values are given in the text.
production. Canine cartilage can only be protected from blood-induced damage when IL-4 plus IL-10 is added within 4 h after the start of blood exposure. Most interestingly, removal of blood from cartilage, mimicking joint aspiration, leads to almost full recovery when performed within 1 day and a partial recovery when performed within 2 days. Subsequent addition of IL-4 plus IL-10 is of no additional value.

Previously we have shown that IL-4 plus IL-10 has added value over just using IL-4 or IL-10 [23]. Moreover, in arthritis conditions, the additive value has also been demonstrated [21]. Therefore, in the present study, the isolated cytokines have not been tested alone.

It takes about 24 h in our human in vitro blood/cartilage culture system before protein levels of IL-1β can be detected. These levels are comparable to unstimulated whole blood culture of healthy controls after 3 days [27]. The onset of IL-1β production can only be blocked within the first 4 to 8 h by addition of IL-4 plus IL-10. When blood is aspirated from cartilage within 24 h, IL-1β is removed from the culture together with red blood cell-derived iron, thereby preventing cartilage damage. Even when catalytic iron reaches the chondrocytes, protein levels of IL-1β within the first 24 h of culture are still too low to enhance hydrogen peroxide production by chondrocytes. As such, the effect of IL-4 plus IL-10 on the inhibition of IL-1β production by monocytes/macrophages is the main reason for the protective effect of this cytokine combination on blood-induced cartilage damage. However, it is plausible to expect additional small direct positive effects of IL-4 plus IL-10 on cartilage chondrocytes, as has been suggested [28]. Upregulation of IL-4 and IL-10 receptor expression on chondrocytes in cartilage tissue upon blood exposure has been found previously [23], although it was demonstrated that human chondrosarcoma cells (SW1353) do not express functional IL-10 receptors [29].

It was considered of relevance to study the effects of IL-4 plus IL-10 on canine blood-exposed cartilage as well, since this is a model frequently described [24, 30–34]. This study shows that IL-4 plus IL-10 needs to be administered more quickly after blood exposure of canine cartilage than in the case of human cartilage. It could be that the thickness of the cartilage influences the degree of blood-induced cartilage damage. Canine hip cartilage is about 1.2 mm thick [35], while cartilage of the centre of the human humeral joint surface, as used in this study, is about 2.2 mm thick [36]. Chondrocytes in thinner cartilage are probably more easily exposed to IL-1β and free iron.

Shortening the time of blood exposure by removal of blood from the cartilage has been shown to be very effective. This is the first time that aspiration has been studied in a clinically relevant setup, as only most of the blood was removed from the cartilage, not all. This mimics human joint aspiration where, even when a joint is washed out, blood will always be left in the joint. The outcome of this study is confirmed by previous research indicating that a longer exposure time results in more prolonged damage [37]. Moreover, removal of intra-articular blood during surgery, as demonstrated in an experimental canine model of anterior cruciate ligament transaction, results in less synovitis and synovial iron deposits [2]. As such, in the case of traumatic joint bleeds and in haemophilia, aspiration of blood from the joint within 24 h is advocated to prevent prolonged damage over time.

Upon removal of blood from the joint, the addition of IL-4 plus IL-10 is of no additive value. This suggests that in clinical practice, administration of IL-4 plus IL-10 after aspiration is of no supplementary value. However, due to joint compartmentalization, some blood may remain after aspiration in clinical practice, on which IL-4 plus IL-10 can have a protective effect. Moreover, it has to be kept in mind that in this study only the direct effect of blood on cartilage was studied. Besides cartilage damage, intra-articular blood also causes inflammation of synovial tissue in vivo [38, 39]. It has been shown in arthritis in vitro [40] and in vivo [41–44] models that administration of IL-4 and IL-10 inhibits inflammation, indicating that IL-4 plus IL-10 in addition to aspiration can have beneficial effects in vivo on the reduction of synovial inflammation.

The present study clearly demonstrates that, despite their cartilage protective properties, there is only a short time window after the onset of a joint bleed in which IL-4 plus IL-10 can limit blood-induced cartilage damage. Aspiration of a joint to shorten blood exposure time is the most effective treatment option and needs to be advocated. Even though it was not demonstrated in these in vitro cartilage culture experiments, the combination of aspiration with IA injection of IL-4 plus IL-10 might help to counteract inflammatory activities induced by a joint bleed and may aid in the prevention of harmful effects of blood on cartilage.

### Rheumatology key messages

- The time window for IL-4 plus IL-10 to protect against blood-induced cartilage damage is short.
- Aspiration of blood from cartilage prevents blood-induced cartilage damage.

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### Disclosure statement

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