CLINICAL STUDY

Androgen receptor CAG repeat polymorphism is associated with serum testosterone levels, obesity and serum leptin in men with type 2 diabetes

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Abstract

Objective: To determine the relationships between androgen receptor CAG repeat polymorphism length (AR CAG), sex hormones and clinical variables in men with type 2 diabetes (DM2). Men with DM2 are known to have a high prevalence of low testosterone levels. Studies suggest that testosterone replacement therapy may improve insulin sensitivity and glycaemic control in men with DM2 and reduces central obesity and serum leptin. AR CAG is known to correlate negatively with AR sensitivity and positively with body fat, insulin levels, and leptin in healthy men.

Design: Cross-sectional study set in a district general hospital diabetes centre.

Methods: Sex hormones, AR CAG and symptoms of hypogonadism were assessed in 233 men with DM2. Associations were sought between these variables and others such as obesity, leptin, glycaemic control, and blood pressure.

Results: Testosterone was negatively associated and AR CAG positively associated with obesity and leptin. The associations of AR CAG with leptin and obesity were independent of testosterone, estradiol, gonadotropins, and age. AR CAG was also independently associated with total, bioavailable and free testosterone, LH, waist circumference, body mass index, leptin, and systolic blood pressure. There was no association of AR CAG with sex hormone binding globulin, estradiol, HbA_{1c} or the symptoms of hypogonadism.

Conclusions: The association of longer AR CAG with obesity and leptin suggests that shorter AR CAG may have an influence in maintaining healthy anthropometrics and metabolism in men with DM2. Testosterone and LH levels are higher in men with longer AR CAG, probably reflecting reduced negative feedback through a less sensitive receptor.

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Introduction

Important functions of testosterone in modulating adiposity, insulin resistance, and type 2 diabetes (DM2) have been postulated (1). It has been known for some time that total testosterone (TT) and sex hormone binding globulin (SHBG) levels are lower in men with DM2 compared with healthy controls (2). A recent study from our research group confirmed a high prevalence of low serum testosterone, including bioavailable (BioT) and free testosterone (FT), in this patient group (3). Furthermore, the clinical syndrome of hypogonadism was commonly present. FT and BioT measures of androgen status are important, as they reflect testosterone availability to target tissues. Results from longitudinal studies have shown that low testosterone levels predict the future development of the metabolic syndrome and DM2 (4–8). Clinical trials have demonstrated that testosterone replacement therapy (TRT) reduces fat mass and central

obesity, and some pilot studies have shown improved insulin sensitivity and glycaemic control in patients with or without DM2 (9, 10). Results from larger multi-centre trials are awaited. Leptin is produced from adipose tissue and has important roles in energy balance including appetite control. Obese individuals are resistant to the effects of leptin and have high levels of the hormone (11). Leptin levels fall during TRT in hypogonadal men with and without DM2 (10, 12).

The actions of androgens including testosterone, on target cells have conventionally been thought to occur via association with the androgen receptor (AR). This is a high affinity nuclear receptor which acts as a transcription factor after association with an appropriate ligand. The AR gene is located on the long arm of the X-chromosome. Exon 1 of the AR gene contains a polymorphic CAG repeat sequence which encodes a variable length polyglutamine stretch (AR CAG) (13). The length of this sequence correlates with the

transcriptional capacity of the AR so that longer sequences are associated with impaired transcriptional activity (14, 15). Studies have shown that the AR CAG polymorphism is clinically relevant. Shorter AR CAG are associated with prostate cancer (16, 17), benign prostatic hypertrophy (18, 19), prostate growth during testosterone treatment (20), semen parameters of fertility (21–23) and bone mineral density (24). In men with Klinefelter's syndrome, longer AR CAG are associated with gynaecomastia and smaller testes (25). The role of AR CAG in determining adiposity, insulin resistance and cardiometabolic risk is therefore of great interest. AR CAG greater than 37 are pathological and cause the rare inherited neurodegenerative disease bulbar muscular atrophy also known as Kennedy syndrome (26). Non-neurological features of Kennedy syndrome include varying degrees of androgen insensitivity with gynaecomastia or testicular atrophy. A link between carbohydrate metabolism and androgenicity is supported by the association of Kennedy syndrome with DM2 (26). A study of AR CAG in healthy male volunteers found that shorter repeat sequences were associated with lower serum insulin and leptin levels and lower fat mass (27). To our knowledge there have been no previous studies of AR CAG in men with diabetes.

Some studies have found correlations between testosterone levels and AR CAG in healthy men. A study of patients from the Massachusetts Male Aging study found that TT and FT levels were positively associated with AR CAG and that the decrease in testosterone levels with aging is greater in patients with a shorter repeat sequence (21). Studies of other male populations have not confirmed a significant effect of AR CAG repeat length on serum testosterone levels (28–30). Testosterone can also have actions independent of the AR. This can occur after metabolism of testosterone to another active hormone, for example conversion to estradiol under the action of the enzyme aromatase. There is now also evidence that testosterone can act in a non-genomic way at the cell surface, for example causing vasodilatation via blockade of L-type calcium channels in vascular smooth muscle cells (31).

This study investigates the relationship of AR CAG with obesity, leptin, blood pressure, glycaemic control, testosterone levels and hypogonadal symptom scores in a group of 233 men. These men were recruited from an original study of 355 men with DM2 which described a high prevalence of hypogonadism in this group and a negative correlation of testosterone with body mass index (BMI) and waist circumference. Our primary hypothesis was that AR CAG would be associated with obesity such that longer AR CAG with less transcriptional activity would be associated with greater BMI and waist circumference (3), which would be in line with the findings for testosterone. Secondary hypotheses were that AR CAG was associated with the other measured variables and that testosterone levels would also be associated with these variables.

Materials and methods

Patients

An initial cross-sectional study of 355 men over the age of 30 with DM2 was conducted at Barnsley Hospital NHS Foundation Trust, Barnsley, UK (3). Subjects were recruited from their appointments at the Centre for Diabetes and Endocrinology and gave informed consent. Many of the subjects were recruited from the retinal screening programme. The study population contained patients with diabetes usually managed in primary, as well as secondary care. One man was of Arabian heritage, all the others were white Caucasian.

At the time of recruitment to the trial details of demography, medical history and drug histories were collected using a questionnaire. Clinical and biochemical assessments of androgen status were made. Other measurements included blood pressure, height, weight, waist and hip circumference. Blood and serum samples were saved for potential future analysis.

Subsequently consent was sought from the initial study population to measure AR CAG and 233 men gave permission. The study was granted ethical approval by Barnsley Local Research Ethics Committee.

Assessments

Assessments took place in the morning between 0800 and 1000 h. Patients were assessed for hypogonadism. They completed the Androgen Deficiency in the Aging Male (ADAM) questionnaire which is validated to assess hypogonadism in aging males and comprises ten questions (32). Venous blood was taken and serum samples produced by centrifugation. Whole blood anticoagulated with EDTA and serum samples were then stored at -20°C for future analysis. Serum TT, SHBG and estradiol were measured by ELISA using a commercially available kit (DRG diagnostics, Marburg, Germany). BioT was determined by a modification of the ammonium sulphate precipitation method described by Tremblay & Dube (33). FT was calculated from TT and SHBG by the formula of Vermeulen *et al.* (34). LH and FSH were measured by ADVIA Centaur immunoassay (Bayer HealthCare).

Patients also completed a questionnaire detailing their medical history and current medications. Weight and height were recorded and used to derive BMI. Waist circumference (waist) was measured midway between the lower costal margins and the iliac crests. Blood pressure was recorded. Glycated haemoglobin (HbA_{1c}) was assessed by Menarini Analyzer HA8160 (Menarini Diagnostics, Wokingham, UK).

Serum leptin was measured by ELISA (R&D systems, Minneapolis, MN, USA) in 114 of the participating men. This group was selected at random from the study population.

Molecular study

DNA was extracted from peripheral lymphocytes in whole blood and subjected to PCR to amplify the region of the AR gene containing AR CAG. The primers used for amplification were 5'-GCT GTG AAG GTT GCT GTT CCT CAT-3' and 5'-TCC AGA ATC TGT TCC AGA GCG TGC-3'. DNA was amplified in 25 µl reactions containing 22.5 µl PCR Master mix (ABGene, Epsom, UK- 1.25UDNA polymerase, 75 mM Tris-HCl (pH 8.8 at 25 °C), 20 mM (NH₄)₂SO₄, 0.01% Tween 20c, 200 µM of each dATP, dCTP, dGTP, dTTP, 1.5 mM MgCl₂), 0.5 µl 10 pmol each primer, 0.5 µl distilled water and 1 µl DNA containing sample. Amplifications were performed using an automated thermal cycler applying the following PCR conditions: 94 °C for 5 min, followed by 32 cycles of 1 min at 94 °C, 1 min at 58 °C and 1 min at 72 °C followed by a 7 min final extension at 72 °C and denaturation for 5 min at 96 °C. Following magnetic separation of PCR products each sample was analyzed by the capillary based AB3730 automated sequencer (Applied Biosystems, Warrington, UK) which produces electropherograms from which the DNA sequence could be derived.

Statistical analysis

Statistical analysis was carried out using the SPSS package. All descriptive data are expressed as mean $\pm$ s.d. Data were assessed and found to be of approximate normal distribution. Correlations were assessed and are expressed as a Pearson correlation coefficient (r) with associated P value. Results were adjusted for age which is known to affect serum testosterone and SHBG levels. Linear regression techniques were used to assess the independence of associations between AR CAG and other variables. Results are expressed using the regression coefficient (β) and a significance value (P) and the quality of the entire model for each calculation is recorded (r^2). Further analysis was performed using ANOVA to compare mean values of variables in groups of patients split into quartiles by AR CAG repeat number (AR CAG = 19 or fewer versus 20–21 vs 22–23 vs 24 or more repeats). Student's t -test was used to compare mean values between two groups where the distribution was approximately normal. Results were considered statistically significant at $P < 0.05$.

Results

Population characteristics and association of variables with age

In our population AR CAG ranged from 8 to 38 with an approximate normal distribution, a mean of 21.76 and a median of 21 (see Fig. 1). One man had an AR CAG repeat length of 38. This is potentially pathological for Kennedy syndrome but he exhibits no neurological abnormalities at the current time. He is to be referred for genetic counselling and neurophysiological testing.

Figure 1 The distribution of AR CAG in our population; this shows an approximate normal distribution similar to previous Caucasian populations studied.

We compared our study population with those men who took part in the original study but failed to give consent for inclusion in the AR CAG study. Men who agreed to take part in our study were significantly older and had significantly higher testosterone and lower HbA_{1c} levels than those who did not agree to take part (see Table 1). The groups were similar in terms of obesity, blood pressure, gonadotropins and estradiol levels.

With regard to hypoglycaemic therapies in our population there were 49 men on diabetic diet only, 128 men treated with oral hypoglycaemic agents without insulin and 56 treated with insulin (with or without oral hypoglycaemic agents).

Age was positively correlated with SHBG ($r = 0.421$, $P < 0.001$), LH ($r = 0.224$, $P = 0.001$), FSH ($r = 0.214$, $P = 0.001$) and systolic blood pressure ($r = 0.207$, $P = 0.002$). There were also significant inverse correlations of age with FT ($r = -0.183$, $P = 0.005$), BMI ($r = -0.297$, $P < 0.001$), waist ($r = -0.379$, $P < 0.001$) and leptin ($r = -0.228$, $P = 0.015$). TT, BioT, estradiol and other remaining variables were not associated with age (data not shown).

Association of AR CAG with testosterone, gonadotropins and estradiol

Table 2 shows the Pearson coefficients for relationships between AR CAG, testosterone, estradiol, gonadotropins and ADAM. Longer AR CAG were associated with higher testosterone and LH but not FSH levels. Testosterone was positively associated with estradiol. ADAM result expressed as a score out of ten did not correlate with testosterone, estradiol, gonadotropins or AR CAG repeat length. Linear regression revealed that AR CAG was associated with TT ($\beta = 0.209$, $P = 0.011$, (whole model) $r^2 = 0.09$), BioT ($\beta = 0.239$, $P = 0.001$, $r^2 = 0.105$)

Table 1 Comparison of variables between our study population and those who failed to give consent for inclusion in the genetic study.

Parameter	Units	Study group	Non participants	P
Age	Years	59.6	56.5	0.037
CAG repeat length (AR CAG)		21.8	—	—
ADAM questionnaire score (ADAM)		4.4	4.15	0.403
Total testosterone (TT)	nmol/l	13.3	11.5	0.004
Bioavailable testosterone (BioT)	nmol/l	4.15	3.75	0.027
Free testosterone (FT)	pmol/l	281	260	0.145
Sex hormone binding globulin (SHBG)	nmol/l	34.6	28.4	0.005
Total estradiol (E ₂)	nmol/l	23.9	22.7	0.34
LH	mIU/ml	4.8	5.2	0.412
FSH	mIU/ml	7.2	8.4	0.161
Body mass index (BMI)	kg/m ²	32	32.9	0.205
Waist circumference (Waist)	cm	109.2	110.6	0.376
HbA _{1c}	%	7.1	7.5	0.005
Systolic blood pressure (Sys BP)	mmHg	143.2	143.6	0.823
Diastolic blood pressure (Dias BP)	mmHg	82.3	81.7	0.654

Levels of statistical significance are given as bold and bold-underlined for *P* values below 0.05 and 0.01 respectively (To convert testosterone/SHBG to ng/dl divide by 0.0347. To convert estradiol to pg/ml divide by 3.671).

and FT ($\beta=0.225$, $P=0.005$, $r^2=0.096$) independent of age, waist, BMI, SHBG, estradiol and gonadotropins.

In view of the association of AR CAG with testosterone and LH which was not found in some previous trials we further investigated by considering the data in quartiles. ANOVA confirmed that there were significant changes in BioT ($P=0.014$) and LH ($P=0.019$) across quartiles of AR CAG but changes in TT ($P=0.148$) and FT ($P=0.120$) were not significant. Figure 2 shows mean BioT and LH by quartiles of AR CAG and shows that variability of AR CAG within the lower half of the distribution was not associated with alterations in testosterone or LH levels and that changes only occurred in the upper quartiles. Indeed, patients in the upper quartile of AR CAG had higher BioT (4.6 vs 4.0 nmol/l, $P=0.007$), TT (14.5 vs 12.9 nmol/l, $P=0.046$), estradiol (26.4 vs 22.9 nmol/l, $P=0.043$) and LH (5.7 vs 4.5 mIU/ml, $P=0.008$) levels than the rest of the group. There was a trend to higher FT in the upper quartile of AR CAG which did not reach statistical significance (358 vs 318 pmol/l, $P=0.09$). There were no differences in SHBG and FSH in different quartiles of AR CAG.

Association of AR CAG with obesity and leptin

Table 3 shows the correlations of AR CAG and testosterone with adiposity, leptin, HbA_{1c} and blood pressure in our population. Obesity and leptin were

inversely correlated with testosterone especially BioT and TT. AR CAG repeat length was significantly correlated with BMI and leptin. Linear regression revealed that AR CAG was significantly associated with waist circumference ($\beta=0.145$, $P=0.019$, (whole model) $r^2=0.23$) and BMI ($\beta=0.171$, $P=0.004$, $r^2=0.285$) independent of testosterone, estradiol, gonadotropins and age but testosterone and age were the strongest predictors of obesity in these models (see Table 4). AR CAG was also associated with leptin independent of waist, BMI, testosterone, estradiol, gonadotropins and age ($\beta=0.163$, $P=0.022$, $r^2=0.579$) but testosterone was not independently associated with leptin and the only other significant predictor was waist ($\beta=0.416$, $P=0.011$).

Association of AR CAG with glycaemic control and blood pressure

Systolic blood pressure was positively associated with AR CAG repeat length (see Table 3). Multiple regression showed that this was independent of known confounders such as obesity and age and was also independent of testosterone, estradiol and gonadotropins ($\beta=0.153$, $P=0.028$). A similar pattern was seen for diastolic blood pressure but the result marginally failed to reach significance. Analysis of blood pressure versus AR CAG repeat length in the 71 patients not treated with antihypertensive medication found no significant

Table 2 Pearson coefficients (*r*) for the associations between androgen receptor (AR) CAG repeat length and sex hormone status after adjusting for age.

	TT	BioT	cFT	E ₂	SHBG	ADAM	LH	FSH
AR CAG	0.147	0.176	0.137	0.091	0.07	−0.064	0.176	0.116
TT		0.832	0.793	0.376	0.358	−0.051	−0.066	− 0.163
BioT			0.824	0.359	0.072	−0.059	−0.093	− 0.139

Levels of statistical significance are given as bold, bold-underlined and bold-underlined-italics for *P* values below 0.05, 0.01 and 0.001 respectively.

Figure 2 Error bar plot showing average bioavailable testosterone and LH levels versus AR CAG in quartiles. Bars show 95% confidence intervals. Bioavailable testosterone was stable across lower quartiles of AR CAG but was significantly higher in men with the upper quartile of AR CAG (134 vs 114 ng/dl, $P=0.007$ versus other groups). LH levels were also stable in the lower quartiles but raised in the upper quartile (5.7 vs 4.5 mIU/ml, $P=0.008$ versus other groups).

association with systolic or diastolic values ($r=0.022$ and -0.053 respectively). Blood pressure was not associated with testosterone levels. Glycaemic control as assessed by HbA_{1c} was not associated with testosterone levels or AR CAG polymorphism. Average testosterone levels and AR CAG repeat lengths did not vary between groups of men treated with diet alone versus those treated with oral hypoglycaemics versus those treated with insulin (data not shown).

Discussion

Our study adds to evidence linking low testosterone with central obesity and high serum leptin. It also provides new evidence of the importance of the AR CAG polymorphism within this relationship. We found positive correlations of AR CAG repeat length with measures of obesity and leptin and AR CAG was independently associated with waist circumference, BMI and leptin on regression analysis. Our study also confirmed a negative association of testosterone with BMI, waist and leptin. Interventional trials have shown that testosterone acts to reduce overall fat mass and/or central obesity and leptin (9, 12, 35, 36) and our results are consistent with the hypothesis that testosterone acts via the AR in the modulation of fat distribution

described in these trials. As expected waist circumference was the major determinant of leptin in our population but the finding that leptin is independently associated with AR CAG but not testosterone is intriguing. It could suggest that stimulation of the AR may reduce leptin levels by a mechanism independent of reductions in fat mass but that this effect may be offset by opposing effects of testosterone elsewhere such as after metabolism to other active hormones or via non-AR receptors. Future research using the emerging selective AR modulators will help decipher the effects of AR stimulation from the overall effects of testosterone. In the meantime work with the testicular feminized mouse which exhibits a nonfunctional AR, has shown that this model exhibits greater weight gain and lipid deposition in the aorta than controls, further suggesting a role of the AR in modulation of obesity and atheroma (37).

Obesity was also associated with higher estradiol in our population, reflecting that the primary site of aromatization of testosterone to estrogen is adipose tissue. The association of testosterone, estradiol, LH and AR CAG in this study are in line with the proposed-hypogonadal-obesity-adipocytokine cycle which provides a hypothesis to link low testosterone levels with visceral adiposity and high leptin levels in men (1, 38).

Table 3 Pearson coefficients (r) for the associations between androgen receptor (AR) CAG repeat length, testosterone and other variables after adjusting for age.

	BMI	Waist	Leptin	HbA _{1c}	Sys BP	Dias BP
AR CAG	0.138	0.101	0.207	-0.086	0.14	0.121
TT	-0.212	-0.253	-0.187	-0.093	-0.051	-0.047
BioT	-0.188	-0.246	-0.193	-0.117	-0.036	-0.017
FT	-0.108	-0.17	-0.125	-0.127	-0.029	-0.017
E ₂	0.174	0.129	0.172	-0.103	-0.016	0.027

Levels of statistical significance are given as bold, bold-underlined and bold-underlined-italics for P values below 0.05, 0.01 and 0.001 respectively.

Table 4 Results from linear regression models for waist and body mass index (BMI).

	Waist		BMI	
	$r^2=0.23$		$r^2=0.285$	
	β	<i>P</i>	β	<i>P</i>
AR CAG	0.145	0.019	0.169	0.004
BioT	−0.331	0.007	−0.282	<0.001
SHBG	−0.158	0.021	−0.179	0.007
E ₂	0.229	<0.001	0.249	<0.001
Age	−0.274	<0.001	−0.345	<0.001

*r*² value for the model given under each heading. Standardized regression coefficient and *P* value given for each significantly associated variable. LH and FSH were also included in the models but were not significantly associated with waist or BMI.

AR CAG was found to correlate with testosterone and LH levels and was independently associated with testosterone levels in multiple regression analysis. This is likely to reflect the role of the AR in the negative feedback of testosterone at hypothalamic and pituitary levels. In this hypothesis the less active AR with longer AR CAG produces less suppression of LH release, thereby increasing LH levels and stimulating higher testosterone levels. It should be kept in mind that LH release is pulsatile. Serum samples were collected at one time point only, so that our study may underestimate the power of relationships of LH with other factors. It is interesting that only variations of AR CAG in the upper half of normality affected testosterone and LH levels suggesting a possible threshold effect in the relationship between testosterone and AR CAG. This needs to be evaluated in further studies or existing data sets because it is an unexpected result and there is no known threshold effect of AR CAG on AR transcriptional activity. If confirmed it could explain the inconsistent results of studies analyzing the relationship between AR CAG and testosterone (28–30) and calls into question the validity of using statistical methods designed to detect linear relationships between these variables.

The higher levels of testosterone found in men with less transcriptionally active ARs have the potential to offset the clinical effects of the receptor polymorphism. Some men with hypogonadal range or low-normal testosterone levels may not be able to produce such an increase in testosterone, and it may be in this circumstance that the polymorphism becomes clinically relevant. Higher testosterone levels found in men with a less sensitive receptor may themselves have effects via mechanisms other than the classical AR so that the levels are not truly compensatory (31).

The AR CAG polymorphism in our study population of men DM2, shows a distribution similar to that previously found in Caucasian populations (27, 39). Therefore, it does not suggest that the AR CAG is an important factor in the causation of DM2 at population level. This does not exclude a role for the polymorphism in the pathogenesis of DM2 in the small number of men

with AR CAG in the upper normal range. It is not unexpected that testosterone and AR CAG had no relationship to HbA_{1c} in our population, because patients were variously on treatment with diet, oral hypoglycaemics and insulin with the aim of meeting diabetes treatment targets. Data from healthy men has already shown that serum insulin levels are associated with AR CAG (27). Future directions for study in this area could focus on the potential relationship between AR CAG and the age of individuals at the time of diagnosis of DM2 or the rate of progression to diabetes requiring oral hypoglycaemic or insulin treatment.

The association of AR CAG with blood pressure deserves further attention given that recently presented data from non-diabetic patients by a separate research team described a similar relationship of AR CAG with blood pressure (Zitzmann personal communication). It may be that testosterone and the AR CAG could be linked to hypertension via a shared association with visceral adiposity but there was no evidence of any association of testosterone levels with blood pressure in our population. Alternatively the finding that the association was not present in those patients untreated with antihypertensive medication may suggest a false positive result. This association therefore deserves more study.

One of the main limitations of our study was that most of the participants were treated with medications that could affect BMI, waist, HbA_{1c} and blood pressure levels. These medications could distort associations between AR CAG, androgen status and other variables and it is likely that our study underestimates the effects of the CAG polymorphism for this reason. Another limitation is the absence of a control group of non-diabetic men but our data still allows us to assess the effect of AR CAG within the diabetic population, and the similarity between AR CAG distribution in our population compared to other non-diabetic Caucasian populations is reassuring. It is also important to note that this cross-sectional study can only describe relationships between AR CAG, obesity and other variables and does not provide evidence of causal effects of AR CAG. A further limitation is that around 50% of men in the trial were treated with statins and we recently reported that use of these drugs is associated with lower serum TT and SHBG levels without any changes in bioavailable or FT (40). Our group differed from those men who did not give consent in terms of age, testosterone levels and glycaemic control. The reason for this is unknown but we believe that it is not likely to affect the overall results of the study given that the relationships between testosterone and other variables such as obesity are of a similar nature to those described previously in the whole group (9).

Further cross-sectional studies are required to assess the effects of AR CAG in a variety of populations, whilst assessment in future therapeutic trials of testosterone will also be valuable. Future clinical uses of measuring

AR CAG may be as an adjunct in the clinical assessment of hypogonadism or obesity and possibly in predicting responses to treatment of these conditions. In the meantime it provides a useful research tool providing clues of the actions of testosterone via the classical AR versus other mechanisms. The receptor polymorphism may also prove relevant in defining target testosterone levels for androgen replacement therapy because those men with insensitive receptors may require high-normal serum testosterone levels to optimize benefit.

Declaration of interest

We have no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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