ORIGINAL ARTICLE

Thyroid hormone status within the physiological range affects bone mass and density in healthy men at the age of peak bone mass

Running Title: Thyroid parameters and bone status

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Abstract

Context: The hormonal factors involved in the regulation of peak bone mass in men have not been fully investigated. Aside from gonadal steroids and somatotropic hormones, thyroid hormones are known to affect bone maturation and homeostasis, and are additional candidate determinants of adult bone mass.

Objective: We aimed to investigate between-subject physiological variation in free and total thyroid hormone concentrations, TSH and thyroid binding globulin (TBG) in relation to parameters of bone mass, geometry and mineral density in healthy men at age of peak bone mass.

Design and setting: 677 healthy male siblings aged 25-45 yrs were recruited in a cross-sectional, population-based study. Areal and volumetric bone parameters were determined using DXA and pQCT. Total and free thyroid hormones, TBG and TSH were determined using immunoassays.

Results: Free and total thyroid hormone concentrations were inversely associated with BMD and BMC at the hip and total body (FT3, TT3, TT4) and at the spine (FT3). TBG was negatively associated with BMC and aBMD at all sites. At the radius, cortical bone area was inversely associated with TT3, TT4 and TBG and trabecular bone density was inversely associated with FT4, TT4 and TBG. We observed inverse associations between cortical bone area at the mid-tibia and FT3, TT3, TT4 and TBG. No associations between TSH and DXA- or pQCT-measurements were found.

Conclusion: In healthy men at age of peak bone mass, between-subject variation in thyroid hormone concentrations affects bone density, with higher levels of FT3, TT3, TT4 and TBG being associated with less favourable bone density and content.

Abbreviations: DXA, dual-energy X-ray absorptiometry; FT3, free triiodothyronine; FT4, free thyroxine; TT3, total triiodothyronine; TT4, total thyroxine; TSH, thyroid-stimulating hormone; TBG, thyroid binding globulin; pQCT, peripheral quantitative computed tomography; aBMD, areal bone

mineral density; BMC, bone mineral content; CV, coefficient of variation; SHBG: sex hormone binding globulin.

Introduction

Peak bone mass (PBM) in young adults is a major determinant of bone mass later in life (1). Besides environmental factors such as exercise, smoking, and nutrition, genetic factors play a major role. These genetic and environmental influences are mediated in part by hormonal regulation of bone accrual during growth and maturation (1-3). The major and most extensively studied hormonal systems implicated in this regulation are the somatotropic and the gonadal axes (3, 4). Another candidate hormonal determinant of PBM is thyroid hormone, known to have potentially marked effects on bone maturation and metabolism.

Thyroid hormone is essential for normal growth and bone development. Thyroid hormone deficiency results in delayed skeletal development, delayed bone age and growth arrest accompanied by epiphyseal dysgenesis (5). Hyperthyroidism in childhood, in contrast, induces accelerated skeletal development and growth with advanced bone age, but also early premature fusion of the epiphyseal growth plates and cessation of growth (6).

In adults with hypothyroidism, bone turnover is reduced with a prolonged bone formation phase that leads to an increased mineralization phase and an apparent increase of bone mineral density (BMD). Hyperthyroidism in adulthood, on the other hand, is associated with increased bone turnover and reduction in BMD at various skeletal sites due to increased cortical porosity and accelerated bone loss (7-11). Interestingly, population studies have shown that both hypo- and hyperthyroidism in adults may be associated with an increased fracture risk (12).

However, little is known about the influence of substantial variation in thyroid parameters within the physiological, euthyroid range on bone density and geometry. Two recent studies described the effects of variation across the normal range of thyroid status on areal BMD (aBMD) and fracture susceptibility in middle-aged and older subjects. The first study reported the effects in postmenopausal women (13) and the second study in men and women above the age of fifty five (14). To our present knowledge, no studies have addressed the issue of influence of variation across the normal range of thyroid hormone status on peak bone mass in male. We therefore studied the relationship between

indices of thyroid hormone status within the physiological range and aBMD, volumetric BMD (vBMD) and bone geometry in healthy young male siblings at the age of peak bone mass.

Materials and Methods

Study design and population

Seven hundred sixty seven young men were recruited from 3 semi-rural to suburban communities around Ghent, Belgium. Men aged 25-45 years were contacted by mail and asked if they had a brother in the same age range also willing to participate. The study design and population characteristics have been described previously (3, 4, 15). The general study aim was to investigate the genetic and environmental determinants of peak bone mass in men and therefore we sampled brothers together with their parents. For this publication, we only considered the brothers. After exclusions, 296 pairs of brothers were included in the study. Sixty-four men were included as single participants, when their brother could not participate in the study, 19 men were included as third brother in a family and 2 as fourth brother. All brother analyses were done by family structure. The maximal age difference between brother pairs was arbitrary set at 12 years. All participants were in good health and completed questionnaires about previous illness, lifestyle, physical activity, education, profession, nutrition and smoking. Exclusion criteria were defined as illnesses or medication use affecting body composition, hormone or bone metabolism: current or prolonged (>3 months) use of glucocorticosteroids, anti-androgens, vitamin D supplements, insulin, thyroxin, previous or current use of anti-epileptic drugs, hypogonadism, hyper- or hypothyroidism, cystic fibrosis, malabsorption or eating disorders, disorders of collagen metabolism or bone development, chronic renal failure, alcohol abuse and autoimmune rheumatoid disease. The study protocol was approved by the ethical committee of the Ghent University Hospital and informed consent was obtained from all participants. Smoking habits were registered as current or previous smoking.

Anthropometry and areal BMD

Standing height was measured using a wall-mounted Harpenden stadiometer (Holtain Ltd., Crymuch, UK). Body weight was measured in light indoor clothing without shoes. Anthropometric

measurements were performed as previously described (4) according to the Anthropometric Standardization Reference Manual (16). Areal BMD at the lumbar spine and proximal femur (total hip region) of the non dominant limb was measured using dual energy x-ray absorptiometry (DXA) with a Hologic QDR-4500A device (software version 11.2.1; Hologic, Bedford, MA, USA). The coefficient of variation (CV) % was <1% as calculated from daily spine phantom measurements.

Volumetric BMD and bone geometry

A peripheral quantitative computed tomography (pQCT) device (XCT-2000, Stratec Medizintechnik, Pforzheim, Germany) was used to scan the dominant leg (tibia) and forearm (radius). The dominant side was selected to allow assessment of the relationship between muscle area and bone parameters. The cortical volumetric bone mineral density (vBMD; mg/cm^3), cortical cross-sectional area (mm^2), endosteal and periosteal circumferences, and cortical thickness (mm) were measured at the mid-radius (66% of bone length from the distal end) and mid-tibia (66%). Trabecular vBMD (mg/cm^3) was measured using a scan through the metaphysis (at 4% of bone length) at the non-dominant radius. The CV for the calibration phantom was <1% as calculated form daily phantom measurements.

Biochemical determinations

Venous blood samples were obtained between 08:00 and 10:00 h after overnight fasting. All serum samples were stored at –80°C until batch analysis for parameters of thyroid function and bone metabolism.

Thyroid parameters included thyroid-stimulating hormone (TSH), free thyroxine (FT4), free triiodothyronine (FT3), total T3 (TT3), total T4 (TT4), thyroid binding globulin (TBG) as well as thyroperoxidase antibodies (TPOAb) and thyroglobulin antibodies (TgAb). Parameters of bone metabolism consisted of C-terminal telopeptide of type I collagen (CTX) and procollagen type 1 amino-terminal propeptide (P1NP). Commercial radio-immunoassays were used to determine serum

levels of total T, SHBG (Orion Diagnostica, Espoo, Finnland) and E2 (Clinical Assay; DiaSorin, Saluggia, Italy).

All thyroid parameter measurements with the exception of TT3, TT4 and TBG were performed using an immuno-electrochemiluminescence technique (Modular E 170, Roche Diagnostics GmbH, Mannheim, Germany). This assay has proven to give comparable results with other assays, according to the report from the IFCC WG for standardization of thyroid function tests (17). The intra- and interassay CV % were 1.0 % and 6.1 % for FT4, 4.3 % and 2.9 % for FT3 and 6.7 % and 2.3 % for TSH. TT3, TT4 and TBG were measured using a radioimmunoassay (DIAsource ImmunoAssays S.A., Nivelles, Belgium). The intra- and interassay CV% were 4.7% and 3.7% for TT3, 5.6% and 6.5% for TT4 and 4% and 3.5% for TBG.

Statistics

Descriptives are expressed as mean ± standard deviation or median [1st -3rd quartile] when criteria for normality were not fulfilled (Kolmogorov-Smirnov) and variables (bone parameters, hormone concentrations) were log-transformed in subsequent linear models. Linear mixed-effects modelling was used to evaluate cross-sectional relationships in our study population, taking the interdependence of measurements within families into account. All analyses considering bone mass were adjusted for age, height, weight and smoking status. Parameters of fixed effects were estimated via restricted maximum likelihood estimation and reported as standardized estimates of effect size (β) with their respective standard error. Associations were considered significant at p-values less than 0.05. Statistical analyses were performed using Spotfire S+ 8.1 (Insightful, Seattle, WA, USA) and Medcalc 11 (Mariakerke, Belgium). The formula for R^2 of Cox and Snell was used to calculate the proportion of variation explained by FT3 and TBG.

Results

General characteristics and thyroid hormone status

The general characteristics, thyroid parameters and pQCT-measurements of the whole study population (n=677) are shown in Table 1 and 2. Based on the exclusion criteria, subjects with thyroid disease were excluded a priori. We additionally excluded subjects with positive thyroid auto-immunity after determination of thyroid antibodies (TPOAb>35 U/L or TgAb >115 U/L), leaving 641 subjects. No differences in anthropometrics were observed between subjects with and without thyroid autoimmunity.

TT4 was strongly ($\beta = 0.51 \pm 0.03$, $p<0.0001$) and FT4 was not associated with TBG ($\beta = -0.002$, $p = 0.95$). TT3 was also strongly associated with TBG ($\beta = 0.56 \pm 0.03$, $p<0.0001$), as well as FT3 ($\beta = 0.17 \pm 0.03$, $p<0.0001$). Significant positive associations between free thyroid hormones and ratios of total hormones to TBG were observed ($\beta = 0.24 \pm 0.04$, $p< 0.0001$). No differences in anthropometrics were observed between subjects with and without thyroid autoimmunity.

Thyroid function in healthy young men in relation to age, body composition and life-style factors

No effects of age, body height or weight were observed on TSH, whereas free thyroid hormone concentrations decreased with age (FT4: $\beta = -0.18 \pm 0.04$, $p<0.001$; FT3: $\beta = -0.14 \pm 0.04$, $p<0.001$). TBG concentrations were positively associated with BMI ($\beta=0.13 \pm 0.04$, $p=0.001$)) but not with age. Free T4, but not TT4 concentrations were positively associated with body height ($\beta=0.09 \pm 0.04$, $p=0.03$), whereas no effect of body weight on FT4 or TT4 was observed. FT3 and TT3 were positively related to body weight ($\beta=0.09 \pm 0.04$, $p=0.03$ and $\beta=0.1 \pm 0.04$, $p=0.01$ respectively).

Current smokers displayed higher FT4 (1.49 ± 0.20 ng/dl versus 1.43 ± 0.18 ng/dl, p=0.002), higher FT3 (4.04 ± 0.37 pg/ml versus 3.87 ± 0.35 pg/ml, p<0.001) and lower TSH (1.4 [1.05-1.94] versus 1.53 [1.18-2.06] µU/l, p=0.04) compared to non- or previous smokers.

All further analyses were adjusted for age, body height, weight and smoking.

Thyroid hormone status in relation to areal bone mass (DXA)

We observed inverse associations of both FT3 and TT3 with bone mineral content (BMC) at the spine, the hip as well as the whole body. In addition, areal bone mineral density (aBMD) at both the level of the hip and the whole body was inversely associated with FT3 and TT3. At the level of the spine, aBMD was also inversely related to FT3, but a similar trend for TT3 was only borderline significant (Figure 1, Table 3). FT3 explained 0.52 % of variation of hip BMD and 0.41 % for BMD at the spine, when adjusted for age, length, weight and smoking status.

Likewise, TT4 was inversely associated with BMC at the three measurement sites, and with aBMD at the level of the hip and the total body (Table 3).

There was no significant relation between either FT4 or TSH and BMC or BMD as measured by DXA (Table 3).

We observed a strongly negative association between TBG and DXA parameters at all levels (Table 3). When adjusted for age, length, weight and smoking status, TBG explained 2.6 % of variation in total hip BMD, 0.66 % of variation in BMD at the spine and 1.6 % of variation for the whole body. When introducing TBG in our statistical model as a covariate, only FT3 remained significantly associated with aBMD at spine, hip and whole body, although this association was weakened. The associations between total thyroid hormones and DXA measurements after adjustment for TBG were no longer significant (Data not shown).

Thyroid hormones in relation to bone geometry and volumetric bone density

The associations between thyroid hormones and pQCT-derived bone parameters are shown in Figure 2. At the radius, cortical bone area was inversely associated with TT3 (β= -0.09 ± 0.04, p=0.01), TT4 (β= -0.09 ± 0.04, p=0.01) and TBG (β= -0.12 ± 0.04, p= 0.0008) and trabecular bone density was inversely associated with FT4 (β= -0.08 ± 0.04, p= 0.04), TT4 (β= -0.1 ± 0.04, p= 0.005) and TBG (β= -0.13 ± 0.04, p= 0.001). Inverse associations between cortical bone area at the mid-tibia and FT3 (β= -

0.08 ± 0.04, p= 0.02), TT3 (β= -0.12 ± 0.04, p= 0.0006), TT4 (β= -0.10 ± 0.03, p=0.003) and TBG (β= -0.14 ± 0.04, p= 0.0001) were observed. However, the higher described associations between total thyroid hormones and cortical bone area and trabecular bone density disappeared when TBG was added to the model as a covariate, similar to our observations with DXA-measurements (Data not shown).

No associations between TSH and pQCT-derived bone parameters were observed (Figure 2).

Thyroid hormones in relation to bone turnover markers

There was no relationship between biochemical markers of bone formation (P1NP) or resorption (CTX) and free or total thyroid hormones and TSH (e.g. FT3 and P1NP: p= 0.6). The higher described association between FT3 and aBMD and BMC did not change when adding markers of bone turnover as a covariate (e.g. addition of P1NP: aBMD hip and FT3: p= 0.005, aBMD spine and FT3: p= 0.004, aBMD total body and FT3: p= 0.007).

TBG in relation to sex steroid hormones and SHBG

In order to explore possible mechanisms underlying the association between TBG and bone parameters, we investigated the relationship between sex steroids and TBG. No associations between estrogens (free and total) or testosterone (free and total) and TBG were observed. The associations between TBG and DXA- and pQCT-measurements remained when sex steroids were added to the model (data not shown). The negative associations between TBG and DXA- and pQCT-parameters remained intact when SHBG was added to the model as a covariate.

Discussion

This study demonstrates that between-subject variation in thyroid hormone status within the physiological range is related to areal and volumetric BMD in healthy men at the age of peak bone mass. Higher FT3, TT3, TT4 and TBG are associated with lower aBMD and BMC at various skeletal sites. However, after correction for TBG, only FT3 remains significantly associated with aBMD. Measuring volumetric BMD and bone geometry by pQCT, we observe inverse relationships between FT4, TT4 and TBG and trabecular bone density as well as inverse associations between FT3, TT3, TT4 and TBG and cortical bone area.

The hormonal determinants of bone mass in men have not been fully investigated. Whereas our current understanding on the effects of thyroid hormone status on the skeleton in the adult is merely based on studies in postmenopausal women or in situations characterized by thyroid dysfunction, this is the first study that considers young men at the age of peak bone mass. Moreover, this report describes relations between thyroid status and bone parameters determined not only by two-dimensional areal DXA estimations but also by three-dimensional geometric and volumetric pQCT measurements in men.

Since von Recklinghausen first described a case of thyrotoxic bone disease in 1891, hyperthyroidism is a well known cause of osteopenia and osteoporosis (18). Studies on the relation between hyperthyroidism, aBMD and fracture risk confirm a decrease of aBMD and an increase of fracture risk. This risk augments with advancing age and returns to normal upon treatment of the hyperthyroid state (9, 19, 20). Untreated subclinical hyperthyroidism in postmenopausal women has also been associated with decreased aBMD and a higher fracture risk compared to women who received treatment, although findings have not been unequivocal (21-24). Few data exist on the influence of subclinical hyperthyroidism on the skeleton in men (5).

Thyrotoxicosis results in increased global bone turnover as indicated by elevated values of the biochemical markers of bone formation and bone resorption (5), which in general correlate well with thyroid hormone concentrations, especially in cases of (subclinical) hyperthyroidism (31). Further, histomorphometric data indicate that thyroid hormone excess results in a shortening of the different phases of the remodelling cycle and an approximate 10% relative deficit of bone formation per cycle (8). Therefore, the decrease in BMD observed in thyrotoxicosis is explained both by a (reversible) expansion of the so called remodelling space, with increased cortical porosity, and by accelerated bone loss that might primarily affect trabecular bone. Conversely, hypothyroidism is characterized by a lower global bone turnover and prolongation of the remodelling cycle with a small positive bone balance per cycle. When situations of thyroid hormone excess or deficiency are prolonged, the effects on bone remodelling can potentially result in a decreased or increased mineralization degree of bone tissue, respectively (7, 8) .

The present study differentiates from most clinical data on the relation between thyroid and skeletal status firstly in that we considered young men at the age of peak bone mass and secondly in that we studied the influence of variations in thyroid hormone status within the physiological range. Therefore, we excluded subjects with a history of thyroid disease or treatment with thyroid hormone or with positive levels of thyroid auto-antibodies from our analyses. We observed an inverse relation between FT4 and log TSH in our cohort, but this association was no longer significant after exclusion of subjects with thyroid auto-immunity, compatible with the premise that we studied euthyroid subjects. Our study differs from preceding studies because we also investigated associations between thyroid hormone levels and volumetric bone density and cross-sectional bone geometry. Our data show that thyroid status is not only associated with trabecular bone density, but also with bone size (cortical bone area).

The novelty of our results is that between-subject physiological variation in apparent thyroid hormone exposure is associated with skeletal characteristics in men at the age of peak bone mass, and that

higher levels of thyroid hormones, even within the normal range, have a negative influence on parameters of bone strength at this young adult age. The negative association between thyroid hormone levels and aBMD is seen for all assessed skeletal sites, albeit most consistently for FT3.

In line with our findings in young men, Van der Deure *et al*. reported in elderly men and women a negative association between FT4 and aBMD and, when corrected for BMI, no significant association with TSH although there was a trend (for a positive relation between TSH and aBMD) (14). However, FT3 was not determined in their study. Murphy *et al.* found that both higher FT4 and FT3 were associated with reduced aBMD in euthyroid postmenopausal women (13). We only find associations between FT3 and BMD or BMC, but not with FT4, which is in agreement with the premise that most actions of thyroid hormone in the body are mediated by the active form of thyroid hormone, T3. More specifically in bone, remodelling is considered to be predominantly mediated by via TRα (25).

However, expression of deiodinases in osteoblasts has been demonstrated (26). Not only circulating serum T3 but also local production of T3 in bone can exert effects on bone. Nevertheless, other regulatory mechanisms can play a role and the local production of T3 is dependent on varying concentrations of serum T4. Our data provide evidence for a negative effect of circulating T3 on bone, though this does not exclude local effects of deiodinase activity in bone, aside from the described systemic effects.

Because of the observation of significant negative associations between both total thyroid hormones and TBG and bone parameters, the question raised if TBG could be the main mediator for the associations seen with total thyroid hormones. Indeed, after correction for TBG, the associations between total thyroid hormones and DXA- and pQCT-parameters became insignificant. We hypothesized that the effects of TBG on bone might be mediated by sex steroids, since administration of estrogens and androgens is known to be associated with an increase and decrease in the level of TBG respectively (27). However, in our study, TBG was not associated with levels of free or total

endogenous estrogen or testosterone. Furthermore, the higher described associations between TBG and bone parameters remained significant when sex steroids or SHBG were introduced to our model as covariates. Moreover, the associations of TBG and bone parameters were independent from SHBG and appeared to be associated more strongly to bone than SHBG. Another argument in favour of an independent role for TBG apart from sex steroids is the consistently negative association between TBG and bone parameters, whereas associations between sex steroids (estradiol) or SHBG and bone parameters are merely positive.

Since TBG is negatively correlated with thyroid state (it decreases in hyperthyroidism and increases in hypothyroidism) (28, 29), one would theoretically expect that the observed relationship with bone would be different. Nevertheless, the associations between TBG and bone parameters are in the same direction as the thyroid hormones themselves (negatively), as is the case with SHBG and sex steroids (positively). In this regard, the binding proteins seem to potentiate the effect of their corresponding free hormones. Underlying these observations, a hypothetical transporter, facilitating hormone transfer across cellular membranes could be active. However, no transporter has been described for TBG and the endocytic megalin-carrier pathway for SHBG remains unproven. TBG could also be a marker for nutritional status since we indeed observed a positive relation between TBG and BMI, but a positive relationship between TBG and bone parameters would be expected and malnutrition was not present in our population of healthy young men.

Further studies should be designed to elucidate if TBG is a real determinant of bone mass or only a marker of another unknown determinant affecting bone.

The present association study does not allow to establish a causal relationship or to unravel underlying pathophysiologic mechanisms. In the context of our primary focus of interest, i.e. the determinants of peak bone mass in men, one of the major questions raised by our findings is whether the recorded associations reflect actual effects of thyroid hormone on bone homeostasis in these adult young men and/or earlier effects of thyroid hormone that occurred during bone accrual. An argument in favour of

the latter possibility might be provided by the fact that we could not observe an association between thyroid hormone levels and biochemical markers of bone turnover and no effect of these markers on the relation between thyroid hormones and aBMD. However, values for biochemical markers of bone turnover are rather high, with a broad range in men in their twenties before decreasing to a nadir towards the age of fifty (30, 31). It is thus possible that an effect of thyroid hormone on these variables has been obscured by other, stronger influences. Another argument in favour of effects of thyroid hormone action on bone acquisition and maturation is our observation of associations of thyroid hormone levels with pQCT-derived geometric bone parameters. Changes of the periosteal circumference by periosteal modelling occur essentially and rapidly during growth, but are slow processes in young adulthood (32).

Recently, some authors have challenged the conventional view that skeletal responses to abnormal thyroid status result solely from altered T3 action in the bone and have proposed TSH as a negative regulator of bone turnover (23, 33-37). For example, Morris *et al*. (35) and Kim and co-workers (23) observed associations between low-normal serum TSH levels and osteopenia and osteoporosis prevalence, together with a graded increase in aBMD with increasing TSH in postmenopausal women. Van der Deure *et al.* found in the Rotterdam study - a population of elderly Caucasians - that femoral neck aBMD as well as a DXA derived cortical thickness estimation increased with serum TSH, but this relationship became non significant when corrected for BMI (14). Mazzioti *et al*. observed that TSH values in the lower part of the normal range were associated with vertebral fractures in euthyroid post-menopausal women, independently of FT4, aBMD and age (37). These findings are opposed by results from the Tromsø and the HUNT 2-study, where, as in our population, no relation of TSH with aBMD was observed within the normal range of serum TSH (34, 36).

Moreover, these epidemiologic studies cannot differentiate between the effects of excess thyroid hormone or TSH deficiency since the hypothalamo-pituitary-thyroid axis, with its feedback regulation

remains intact (38). Despite demonstration of TSH-receptor expression in osteoblasts and osteoclasts (33), in vitro studies have not established a clear and consistent role for TSH in bone cells so far. In contrast, elegant studies in mice provide strong arguments against direct effects of TSH on bone. Bassett *et al*. observed that deletion of TRα in mice resulted in decreased T3 action in bone without affecting systemic thyroid status. Delayed ossification and osteosclerosis indicate that responses to reduced T3 action in bone predominate when TSH levels are normal. Similarly, mutation or deletion of TRβ results in increased T3 action in bone and increased levels of TSH. Accelerated ossification and osteoporosis confirms that the effects of T3 excess predominate even when TSH levels are also increased, providing further evidence supporting that skeletal responses to hypo- and hyperthyroidism are mediated by T3 acting via TRα and not by a direct effect of TSH on bone (38). In our cohort of young men, we could not find any relation between TSH and aBMD, BMC or volumetric trabecular and cortical density nor bone geometry, hereby supporting the conventional view.

We can conclude from this study that between-subject variation of thyroid hormones in the physiological range has an effect on bone mass, density and geometry in healthy young men at the age of peak bone mass, with higher levels of FT3, TT3, TT4 and TBG being associated with lower aBMD and BMC at various skeletal sites.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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