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# Is the haematopoietic effect of testosterone mediated by erythropoietin? The results of a clinical trial in older men

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## SUMMARY

The stimulatory effects of testosterone on erythropoiesis are very well known, but the mechanisms underlying the erythropoietic action of testosterone are still poorly understood, although erythropoietin has long been considered a potential mediator. A total of 108 healthy men >65 years old with serum testosterone concentration <475 ng/dL were recruited by direct mailings to alumni of the University of Pennsylvania and Temple University, and randomized to receive a 60-$cm^2$ testosterone or placebo patch for 36 months. Ninety-six subjects completed the trial. We used information and stored serum specimens from this trial to test the hypothesis that increasing testosterone increases haemoglobin by stimulating erythropoietin production. We used information of 67 men, 43 in the testosterone group and 24 in the placebo group who had banked specimens available for assays of testosterone, haemoglobin and erythropoietin at baseline and after 36 months. The original randomized clinical study was primarily designed to verify the effects of testosterone on bone mineral density. The primary outcome of this report was to investigate whether or not transdermal testosterone increases haemoglobin by increasing erythropoietin levels. The mean age ± SD of the 67 subjects at baseline was 71.8 ± 4.9 years. Testosterone replacement therapy for 36 months, as compared with placebo, induced a significant increase in haemoglobin (0.86 ± 0.31 g/dL, $p = 0.01$), but no change in erythropoietin levels (−0.24 ± 2.16 mIU/mL, $p = 0.91$). Included time-varying measure of erythropoietin did not significantly account for the effect of testosterone on haemoglobin (Treatment-by-time: $\beta = 0.93$, SE = 0.33, $p = 0.01$). No serious adverse effect was observed. Transdermal testosterone treatment of older men for 36 months significantly increased haemoglobin, but not erythropoietin levels. The haematopoietic effect of testosterone does not appear to be mediated by stimulation of erythropoietin production.

## INTRODUCTION

Low testosterone has been shown to be independently associated with anaemia in older individuals (Ferrucci *et al.*, 2006). This is not surprising, given the known stimulatory effects of testosterone on erythropoiesis (Shahidi, 1973; Shahani *et al.*, 2009), as reflected by the increase in haematocrit and haemoglobin often observed during testosterone therapy (Molinari, 1982; Jockenhovel *et al.*, 1997; Basaria & Dobs, 1999; Viallard *et al.*, 2000; Calof *et al.*, 2005; Bhasin *et al.*, 2006; Coviello *et al.*, 2008). Furthermore, erythrocytosis is the most frequent adverse event reported in testosterone trials (Viallard *et al.*, 2000; Calof *et al.*, 2005). For this reason, the Endocrine Society guidelines on androgen deficiency syndromes in men recommend monitoring haematocrit 3 months after initiation of testosterone therapy and annually thereafter (Bhasin *et al.*, 2006). However, the mechanisms by which testosterone stimulate erythropoiesis are poorly understood.

Testosterone can act directly on bone marrow, increasing the number of burst forming units and erythropoietin-responsive cells (Moriyama & Fisher, 1975; Coviello *et al.*, 2008; Shahani *et al.*, 2009) and improving intestinal iron absorption (Shahani *et al.*, 2009). It has also been postulated that testosterone induces erythropoietin production (Rishpon-Meyerstein *et al.*, 1968). This concept has been considered as scientific

dogma based on animal and small human studies (Malgor *et al.*, 1968; Rishpon-Meyerstein *et al.*, 1968). However, the assay previously used to measure erythropoietin is now considered inaccurate, because it is indirect and depends on red blood cell turnover. Indeed, more recent studies in young adult subjects using new accurate methods (ELISA double-antibody sandwich method uses recombinant human erythropoietin as standard) did not replicate that finding (Coviello *et al.*, 2008).

## AIM OF THE STUDY

Because of the scant data on the effect of testosterone on erythropoietin, mostly derived in a young adult population, we investigated whether in older men with low-normal levels of testosterone, transdermal testosterone increases haemoglobin by increasing erythropoietin levels.

## MATERIALS AND METHODS

As described elsewhere, healthy men over 65 years of age were recruited by direct mailings to alumni of the University of Pennsylvania and Temple University and by newspaper and television advertisements. The study included men who had a serum testosterone concentration at least one SD below the mean for healthy young men (<475 ng/dL; 16.5 nmol/L). A total of 108 men met these criteria and were randomized to receive a testosterone or placebo patch in a double blind fashion for 36 months. Ninety-six subjects completed 36 months of treatment. Other inclusion and exclusion criteria are described elsewhere (Snyder *et al.*, 1999). The study reported was performed 10 years later on the data and serum specimens of 67 men, 43 in the testosterone treatment group and 24 in placebo group who had sufficient sera remaining for assays of testosterone, haemoglobin, and erythropoietin. Evaluation time points were baseline and 36 months. The flow diagrams for the original RCT and the post hoc data used in the current analysis of the RCT are shown in Fig. 1.

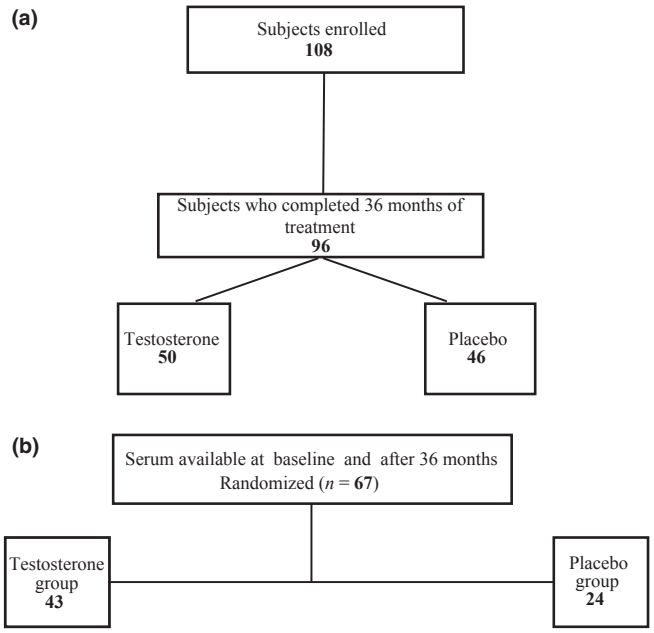
### Study design

Testosterone was administered by scrotal patch (Testoderm; Alza Corp., Palo Alto, CA, USA); placebo patches were identical in appearance to testosterone patches. Each subject was asked to wear a patch at all times except when bathing, change the patch once a day, and shave the scrotum twice a week. Each subject began by wearing a 60-$cm^2$ testosterone patch, which delivers approximately 6 mg testosterone/24 h, or a 60-$cm^2$ placebo patch. The data manager reviewed serum testosterone concentrations every 3 months and directed a decrease in patch size to 40 $cm^2$ if a man's serum testosterone was above 1000 ng/dL (34.7 nmol/L) or re-education in patch technique if a man in the testosterone-treated group had a value less than 250 ng/dL above baseline. To maintain the blinding in both the above manipulations, the data manager directed that a man in the placebo-treated group be treated similarly.

### Storage of the samples

The samples were kept frozen at all times at −80°C. The freeze-thaw cycles that affect some analytes, but not others were very limited.

**Figure 1** Flow diagrams for the original RCT (a) and the post hoc data used in the current analysis of the RCT (b). Ten years after the original study had been completed, a proposal was submitted to National Institute on Aging and the University of Pennsylvania for access to serum available from the study to measure erythropoietin. Of the original 96 subjects, only 67 subjects (43 in the T group and 24 in the placebo group) had serum available from the baseline and 36 month visits. The study was performed on sera from these subjects.




### Hormonal measurements

Total testosterone was assessed in 2008 by electrochemiluminescence immunoassay with minimum detectable concentration (MDC) of 2 ng/dL and interassay coefficient of variation (CV) <10%. Erythropoietin was assessed in 2008 by R&D Systems (Minneapolis, MN, USA) ELISA Human Erythropoietin Quantikine IVD Control 1&2 with MDC of 2.5 mIU/mL and high detection limit of 200 mIU/mL. The estimated normal range of standard was 3.3–16.6 mIU/mL and interassay CV < 10%.

### Statistical methods

Variables were reported as means ± SD, or median and interquartile range, as appropriate. For measures non-normally distributed, log-transformed values were used in the analyses. Differences in baseline characteristics across experimental groups were analysed using independent sample *t*-tests. Changes within groups from baseline to end of treatment were evaluated with paired *t*-tests.

The association between treatment and change of haemoglobin and erythropoietin over time was examined with random coefficient analyses. Random-effects models were valuable in this context, allowing the detection of variation between subjects and autocorrelation between repeated measurements of the same participants over time. Treatment status and time point (T0–T1) were entered as fixed factors; subjects were treated as a random factor and a random intercept was estimated. Time was coded as a categorical variable and treatment-by-time interaction terms were entered in the models to compare the change in the outcome between treatment groups at follow-up. For significant findings effect sizes are given as *r* calculated using

between group *t*-test value at 36 month (Dunlop *et al.*, 1996). All analyses were performed using SAS (v. 9.1; SAS Institute, Inc., Cary, NC, USA). Significance level was set at $p < 0.05$.

## RESULTS

Table 1 shows the baseline characteristics of the study population. The mean age ± SD of the 67 subjects at baseline was 71.8 ± 4.9 years. The testosterone and placebo groups did not differ at baseline in terms of age, haemoglobin and erythropoietin. The testosterone-treated group had lower baseline testosterone levels and BMI than the placebo group, which was of borderline statistical significance ($p = 0.06$ and $p = 0.16$ respectively). As expected, 36 months of testosterone treatment was associated with an increase in testosterone levels (from 488 ng/dL to 689 ng/dL; $p = <0.001$).

The delta change in testosterone levels was 201.2 ± 285.2 ng/dL and 22.7 ± 73.6 ng/dL in the testosterone and placebo groups respectively ($p = <0.001$ for difference) (Table 2). Haemoglobin levels for Treatment and Placebo groups are depicted in Fig. 2.

Testosterone replacement therapy for 36 months was associated with an increase in mean haemoglobin levels of 0.8 g/dL, from 14.7 ± 1.0 g/dL to 15.5 ± 1.4 g/dL (95% CI: 15.2–15.9). This change was statistically significant (Treatment-by-time: β= 0.86, SE = 0.31, $p = 0.01$). At 36 months, the mean haemoglobin concentration in the placebo group was 14.6 (95% CI: 14.1–15.1), not significantly changed from baseline (Table 3). No change was found in the placebo group (Table 3). Effect size *r* for differences in haemoglobin levels at follow-up was 0.33. To test whether or not changes in erythropoietin mediate the relationship between testosterone and haemoglobin, we included a time-varying measure of erythropoietin as a covariate in the previous mixed model. Results were substantially unchanged (Treatment-by-time: β = 0.93, SE = 0.33, $p = 0.01$). Erythropoietin levels for treatment and placebo group at baseline and after 36 months of testosterone therapy are depicted in Fig. 3.

Testosterone replacement therapy for 36 months did not induce an increase in erythropoietin levels (15.2 ± 19.3–13.8 ± 9.7 mIU/mL). No change occurred in the placebo group either (Table 3) (Fig.3). The change in erythropoietin levels during treatment did not differ between subjects taking testosterone replacement therapy and those taking placebo (Treatment-by-time: β = −0.24, SE = 2.16, $p = 0.91$). When the analysis was adjusted for baseline testosterone levels, results were substantially unchanged.

## DISCUSSION

Transdermal testosterone treatment of elderly men for 36 months significantly increased their haemoglobin concentrations, but not erythropoietin concentrations. The increase of about 1 g/dL in haemoglobin concentration during testosterone treatment might be clinically relevant, especially in older individuals, where anaemia has been associated with poor clinical outcomes (Woodman *et al.*, 2005).

**Table 1** Characteristics of study participants at baseline according to treatment group.

| | Treatment ($n = 43$) | Placebo ($n = 24$) | *p* |
|---|---|---|---|
| Age (years)[a] | 71.8 ± 4.7 | 71.8 ± 5.4 | 0.99 |
| BMI (kg/m²)[a] | 25.8 ± 3.3 | 24.8 ± 2.4 | 0.16 |
| Testosterone (ng/dL) [b] | 402.2 (245.6) | 372.1 (79.1) | 0.06 |
| Creatinine (mg/dL) [b] | 1.10 (0.20) | 1.20 (0.30) | 0.37 |
| Haemoglobin (g/dL) [b] | 14.9 (1.4) | 15.0 (1.7) | 0.84 |
| Erythropoietin (mUI/mL)[b] | 11.7 (7.8) | 11.9 (9.6) | 0.77 |

[a]Means ± SD. [b]Medians ± Interquartile range.

**Table 2** Serum testosterone concentrations (means ± SD and delta) by treatment group at baseline and 36 months.

| | Baseline[a] | 36 months | Delta | *p*[b] |
|---|---|---|---|---|
| T ($N = 43$) | 488.2 ± 230.5 | 689.4 ± 307.3 | 201.2 ± 285.2 | <0.001 |
| Placebo ($N = 24$)[a] | 392.54 ± 83.9 | 415.3 ± 109.1 | 22.7 ± 73.6 | 0.41 |

[a]The baseline values were the means of three different measurements. [b]The *p* values compare the mean change from 0 to 36 months between the two treatment groups.

**Figure 2** Haemoglobin levels for treatment and placebo group at baseline and after 36 months of testosterone therapy.

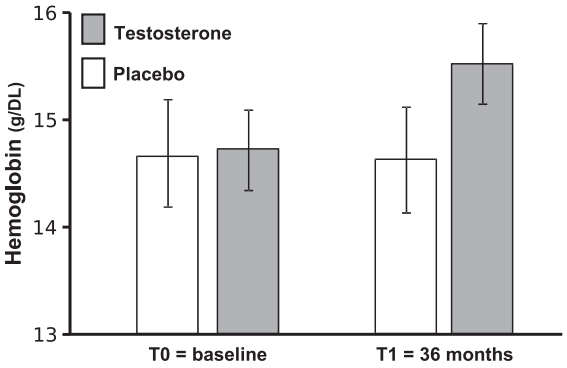
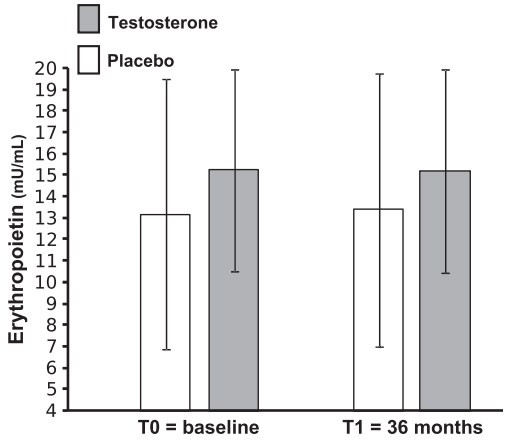



**Table 3** Haemoglobin and erythropoietin concentrations (mean ± SD and change from baseline, delta) by treatment group.

| | Baseline | 36 months | Delta | *p*[b] |
|---|---|---|---|---|
| Haemoglobin (g/dL)[a] | | | | |
| Testosterone | 14.7 ± 1.02 | 15.5 ± 1.36 | 0.80 ± 1.24 | <0.001 |
| Placebo | 14.7 ± 1.44 | 14.6 ± 1.06 | −0.06 ± 1.21 | 0.38 |
| Erythropoietin (mUI/mL)[a] | | | | |
| Testosterone | 15.2 ± 19.32 | 15.4 ± 17.5 | −0.08 ± 9.1 | 0.58 |
| Placebo | 13.2 ± 6.95 | 13.8 ± 9.7 | 0.13 ± 6.1 | 0.59 |

[a]Means ± SD. [b]The *p* values compare the mean change from 0–36 months between the two treatment groups.

**Figure 3** Erythropoietin levels for treatment and placebo group at baseline and after 36 months of testosterone therapy.



Moreover, increases in haemoglobin and haematocrit are also known pharmacological class effects of testosterone. In a meta-analysis of 51 studies of testosterone treatment using different preparations of testosterone, treatment was associated with a significant increase in haemoglobin (weighted mean difference (WMD), 0.80 g/dL; 95% CI, 0.45–1.14) (Fernández-Balsells *et al.*, 2010), an increase of similar magnitude to that seen in the present study. Individual studies also show a similar increase. In a study in which patients were randomized to receive a testosterone gel or patch or a placebo, both testosterone preparations increased haemoglobin and haematocrit more than placebo after 90 days (Steidle *et al.*, 2003). In a study of a testosterone patch, testosterone treatment increased haemoglobin more than placebo (Merza *et al.*, 2006). The mechanism by which testosterone stimulates erythropoiesis, however, is not known. A study performed in five subjects published decades ago reported an increase in serum erythropoietin in response to testosterone and it is the study most cited to address the mechanism of the erythopoietic effect of testosterone. A study in five patients decades ago reported an increase in serum erythropoietin in response to testosterone, but the method used to measure erythropoietin then is not now considered to be reliable (Rishpon-Meyerstein *et al.*, 1968).

Rishpon-Meyerstein measured the plasma concentration of erythropoietin by using a murine bioassay based on the uptake of iron by the red cells of newborn mice. Erythropoietin is currently measured, by us and others (Coviello *et al.*, 2008), using a Quantikine ELISA double-antibody sandwich method that employs recombinant human erythropoietin as standard. These results are consistent with two other studies conducted in young adult and older men (Coviello *et al.*, 2008; Ip *et al.*, 2010). These men were made severely hypogonadal by administration of a GnRH agonist and then treated with graded doses of testosterone. Testosterone increased the haemoglobin concentration in a dose-dependent manner, more in older than younger men, but did not affect serum erythropoietin concentrations. Similar results were observed in a study of 158 men with primary or secondary hypogonadism treated with testosterone pellets for an average of 7 years (Ip *et al.*, 2010). The mean change in serum haematocrit was associated with the mean change in testosterone concentration, while the mean change in serum erythropoietin was not, suggesting that the increase in haematocrit was independent of erythropoietin (Ip *et al.*, 2010).

Recent studies suggest other possible mechanisms by which testosterone could increase erythropoiesis. One study showed that androgens can modify the transcription of the erythropoietin receptor, leading to erythropoietin-initiated actions (Pelekanou *et al.*, 2010). Another study demonstrated a possible role of hepcidin as mediator of the effect of testosterone and haemoglobin (Bachman *et al.*, 2010).

On the basis of our findings we cannot exclude that testosterone affects the biological activity of erythropoietin in ways independent of its production. In vitro studies have shown that testosterone may increase the expression of erythropoietin receptor in the colony forming units-erythroid (CFU-E) (Chateauvieux *et al.*, 2011). In accordance, a recent study showed that membrane-acting androgens can modify the transcription of the erythropoietin receptor, leading to erythropoietin-initiated actions (Pelekanou *et al.*, 2010). These studies support the interesting hypothesis that testosterone increases the biological activity of erythropoietin without affecting erythropoietin concentrations.

Consistent with the notion that haematopoietic actions of androgens are erythropoietin-independent are findings from two population-based studies of men with (Bhatia *et al.*, 2006; Grossmann *et al.*, 2009) and without diabetes (Ferrucci *et al.*, 2006) in whom low testosterone remained a risk factor for anaemia independent of erythropoietin levels. One study in men undergoing haemodialysis found that concurrent androgen therapy not only augmented the anti-anaemic effects of erythropoietin but also reduced the erythropoietin dose necessary to maintain the target haemoglobin level (Ballal *et al.*, 1991), but in another study it did not (Brockenbrough *et al.*, 2006). In a related study in non dialyzed patients with chronic kidney disease, low serum endogenous serum testosterone concentrations were associated with higher requirements for erythropoiesis-stimulating agents than normal testosterone concentrations (Carrero *et al.*, 2011).

The present study has some limitations. The serum samples had been frozen for more than 10 years, and there are no data directly addressing the erythropoietin stability in the freezer. However, the samples were stored at −80°C at all times, and freezing and thawing was kept to a minimum. In addition, there are several reasons to think that erythropoietin is stable for this length of time. The company that provided the erythropoietin kits assessed several proteins for stability and has not uncovered any evidence of degradation in the freezer. Erythropoietin itself seems to be a reasonable stable protein; one study found excellent stability during storage at refrigerator temperatures for 6 weeks (Naughton *et al.*, 2003). Epoietin alfa has been shown to be stable in syringes for 3 and 6 weeks. In addition, many proteins have been studied in samples frozen for considerably longer than 10 years. Measurement of c-reactive protein in the Honolulu Heart Program showed similar results in samples stored for up to 26 years as those collected more recently. Despite these considerations, the possibility that erythropoietin may have degraded over time and that this may have affected our findings cannot be excluded.

A second limitation is that the original study was not designed to evaluate the effect of testosterone on haemoglobin and erythropoietin, but to address its effects on bone density and body composition. We cannot exclude that the study could have been underpowered to detect any significant change in erythropoietin levels.

Third, the mean serum testosterone concentration at baseline was at the low end of the normal range, so testosterone may not have had as much of an effect as if the men had been severely hypogonadal. However, haematocrit and haemoglobin did increase, but erythropoietin did not, again suggesting that the mechanism did not involve erythropoietin. Finally, information on iron status was missing although no patient at baseline was anaemic.

These limitations are offset by important strengths. This is the first randomized, controlled trial to test the effect of testosterone treatment on erythropoietin. Serum erythropoietin levels, available at two time points, were measured by a method now considered accurate. Finally, the results obtained by the random effect model should better explain the within and between participants variation in the outcomes.

In conclusion, transdermal testosterone treatment of older men for 3 years significantly increased the haemoglobin

concentration, but not the erythropoietin concentration, suggesting that testosterone acts by a different mechanism.

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Authors' responsibilities were as follows—Marcello Maggio, Luigi Ferrucci and Peter Snyder: conceived this specific hypothesis of the study; Luigi Ferrucci applied for the funding; Yuri Milaneschi: conducted final statistical analyses; Marcello Maggio: wrote the first draft of the manuscript; All authors: contributed to subsequent drafts of the manuscript, and approved the final version of the manuscript.

The corresponding author (Marcello Maggio) had full access to all data and had final responsibility for the decision to submit the manuscript for publication.

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