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Effects of androgen deprivation therapy on telomere length.

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Short title: androgen deprivation and telomeres

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31

32 **Abstract**

33 Objective: Recent evidence suggests that androgens either directly or via aromatisation
34 to oestradiol may regulate telomere length, hence providing a mechanism whereby
35 reproductive steroids are linked to biological aging in men. Using men with prostate
36 cancer initiating androgen deprivation therapy (ADT), we tested the hypothesis that
37 severe sex steroid deprivation would accelerate telomere shortening.

38 Design: We conducted a secondary analysis of a 2-year prospective controlled study
39 among 65 men with non-metastatic prostate cancer newly commencing adjuvant ADT
40 (n=40) and age- and radiotherapy-matched prostate cancer controls (n=25).

41 Methods: We measured leukocyte telomere length (LTL) expressed as telomeric/single
42 copy control gene (T/S) ratio at baseline, 6, 12 and 24 months. Generalised linear
43 models determined the mean adjusted difference (MAD) [95% confidence interval]
44 between groups during follow-up.

45 Results: Compared to controls over 24 months, men receiving ADT had no change in
46 LTL, MAD for T/S ratio (0.105 [-0.004; 0.213], p=0.235).

47 Conclusions: Using men with prostate cancer receiving ADT as a model we found no
48 evidence that prolonged and profound sex steroid deprivation is associated with
49 accelerated telomerase shortening. Larger studies will be required to confirm, or refute
50 these findings.

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57 **Introduction**

58 Telomeres are specialised hexanucleotide repeats complexed to proteins. They are
59 located at ends of linear chromosomes and protect their structural integrity [1]. Telomere
60 length reduces with increasing chronological age and with accumulation of age-related
61 comorbidities. Given that telomere shortening contributes to cellular senescence,
62 telomere length represents a cellular marker of biological aging [1]. Recent preclinical,
63 observational and interventional studies suggest that androgens play a role in

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preserving telomere length [2-5]. However, whether this is an androgen receptor mediated effect, or whether androgens act indirectly, via aromatisation to oestradiol [6, 7], is unclear.

Because prostate cancer is an androgen responsive malignancy, androgen deprivation therapy (ADT, defined here as medical castration using gonadotropin-releasing hormone (GnRH) analogs) is an effective treatment. ADT reduces serum testosterone, but also oestradiol levels to castrate range. ADT is often prescribed for extended times (*e.g.* 3 years for high-risk prostate cancer with curative intent). Therefore, these patients offer a unique model of profound and global sex steroid deficiency over an extended period [8]: ADT represents the only situation where an ethical requirement for T replacement is absent for prolonged periods. Given that ADT reduces both circulating testosterone and estradiol to near castrate levels, effects on telomere length -if testosterone indeed plays a role- should be evident irrespective of whether this occurs as a direct effect or via its aromatization to estradiol. Moreover, ADT also accelerates certain features associated with biological aging, such as loss of bone [9] and muscle mass [10], and increased insulin resistance [11]. We therefore conducted a secondary analysis of a 2-year, prospective case-control study in men with prostate cancer initiating ADT adjuvant to radiotherapy and age- and radiotherapy-matched prostate cancer controls to test the hypothesis that sex steroid deprivation accelerates telomere shortening.

Subjects and Methods

We conducted a prospective 24-month case-control study at a tertiary referral hospital (Austin Health, Melbourne, Australia). The study was approved by the Human Research Ethics Committee, Austin Health. All participants provided written informed consent. This is a secondary analysis assessing the effects of ADT on telomere length. The effects of ADT on muscle function, the primary outcome of the study are reported elsewhere [12].

Participants were recruited from prostate cancer outpatient clinics. Inclusion criteria included age 55-85 years, localised non-metastatic prostate cancer (Stage T1-3, Nx, M0), and an Eastern Co-operative Oncology Group performance status of 0 (fully active and unrestricted in physical activity). Exclusion criteria included that any illnesses or other

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97 factors predisposing them to androgen deficiency, previous ADT, or significant medical
98 comorbidities including active renal, liver, cardiac, respiratory or joint or
99 neuromuscular disease. Cases were newly commencing long-term ADT with different
100 brands of GnRH agonists, including triptorelin, goserelin, eligard and lucrin, at the
101 discretion of the treating physician. To assess the specific effects of ADT, cases and
102 controls were matched for age, body mass index, medical co-morbidities, radiotherapy
103 treatment and baseline testosterone level [12].
104
105 Blood was drawn in the morning and in the fasted state at 0, 6, 12 and 24 months.
106 Serum total testosterone was measured with a electrochemiluminescence immunoassay
107 using Cobas C8000, Roche Diagnostics (minimum detection 0.4 nmol/l, inter-assay
108 variation 5.0-6.9%), as described [12]. Oestradiol was measured by
109 electrochemiluminescence immunoassay using the same system (minimum detection
110 19.0 pmol/l, inter-assay variation 1.9-3.5%).
111
112 Leukocyte telomere length (LTL) was measured from leukocyte DNA samples by a
113 multiplex quantitative PCR method as described [5]. We optimized a PCR-based
114 methodology for accurate measurement of LTL using the protocol described by
115 Cawthon [13] which we have further developed [5]. Briefly, telomere lengths of leukocyte
116 DNA samples were measured by a multiplex quantitative PCR method. Each sample was
117 amplified for telomeric DNA and for beta globin, a single-copy control gene, which was
118 used as an internal control to normalize the starting amount of DNA. The K562 cell line
119 was used as a standard [14]. A four-point standard curve derived from the K562 cell line
120 was included in each run to assess and compensate for interplate variations in PCR
121 efficiency. The mean PCR efficiencies for telomeric DNA and beta globin are 94.5% and
122 92.4% respectively. Two quality-control samples were also included in each run to
123 assess interplate and intraplate variability of threshold cycle (Ct) values. Furthermore,
124 5% of the test samples were repeated on a different run to account for interplate
125 variability. Periodic reproducibility experiments were performed to confirm adequate
126 normalization. All samples, standards, and controls were run in triplicate and the
127 median value used for analyses. A standard curve derived from K562 cell line was used
128 to transform the cycle threshold into nanograms of DNA. The amount of telomeric DNA
129 (T) was divided by the amount of single-copy control gene DNA (S), producing a relative

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130 measurement of the telomere length (T/S ratio), representing the normalised quantity
131 of telomeric DNA. The coefficient of variation for the quantitative PCR across batches
132 was <10%.

133 <u>Statistical analysis</u>

134 Data were not normally distributed and are presented as median and interquartile
135 range (IQR). Comparisons of baseline characteristics were made using Wilcoxon rank
136 sum test for continuous variables or chi square test for frequencies. Two sided p values
137 <0.05 were considered significant. Repeated measurements were compared between
138 groups using a linear mixed model [15]. The effect of interest was the interaction of time
139 points and group, incorporating baseline values as a fixed covariate and repeated
140 measure by subject as random effect. The model is also robust against regression to the
141 mean. As a quantitative measure, mean adjusted difference (MAD) plus 95% CI between
142 the groups from baseline to 24 months is provided. Statistical analyses were performed
143 using R statistical package (version 3.3.2 for Mac) [16].
144

145 **Results**

146 *Baseline characteristics of the study subjects*

147 Study participants were matched for age, body mass index, medical co-morbidities,
148 radiotherapy and baseline testosterone level (Table 1). Given ADT is added to
149 radiotherapy as treatment for high-risk disease, whereas radiotherapy alone is
150 indicated for intermediate risk disease, baseline Gleason scores and PSA levels were
151 higher in cases compared to controls. At baseline, all men were clinically eugonadal and
152 had age-appropriate normal testosterone levels. There was no difference in baseline
153 LTL, measured by the T/S ratio between cases and controls (Table 1).
154

155 While serum total testosterone and oestradiol levels remained stable in controls, both
156 were decreased to castrate levels in men receiving ADT over the course of the study
157 (Figure 1). Only men recruited relatively early in the study had assessments at 24
158 months, because men recruited al later time points were not followed beyond 12
159 months due to funding limitations.
160

161 *Change in leukocyte telomere length over time*

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162 There was no difference in the LTL, measured by the T/S ratio and presented as mean
163 adjusted differences at each time point of follow-up (6, 12 and 24 months) between
164 men receiving ADT and the age- and radiotherapy-matched prostate cancer, overall
165 p=0.235 (Table 2, Figure 2).
166
167 **Discussion**
168 In this controlled prospective study, using men with prostate cancer receiving ADT as a
169 model, we found no evidence that severe sex steroid deprivation over 2 years is
170 associated with shorter telomere length.
171
172 Our analysis was prompted by recent evidence that sex steroids may modulate telomere
173 length, a marker of cellular senescence, hence providing a potential mechanisms linking
174 decreasing levels of circulating sex steroids to biological aging in men. In a recent cross-
175 sectional study of community dwelling men, we reported that circulating levels of the
176 testosterone metabolites dihydrotestosterone (DHT) and oestradiol, but not
177 testosterone itself, correlate with LTL independently of age [5]. In experimental studies
178 using a variety of cultured cells, testosterone [3], synthetic androgens [2] and oestradiol [2, 6, 7]
179 have been reported to up-regulate telomerase activity, the enzyme that counters
180 telomere shortening. This occurs by sex steroids increasing the expression of *TERT*, the
181 gene expressing the catalytic subunit of telomerase [2, 6, 7]. This effect may involve the
182 activation of an estrogen-responsive element in the reporter region of the telomerase
183 gene [2, 6]. Therefore, based on these preclinical studies, it has been assumed that the
184 aromatisation of androgens to oestradiol is important for telomerase up-regulation.
185 This is consistent with our observational Mendelian randomisation study reporting an
186 association between aromatase gene polymorphisms reducing serum oestradiol and
187 shorter LTL in community dwelling men [5]. However, in a recent phase 1-2 prospective
188 study, danazol treatment, a non-aromatisable androgen that cannot be converted to
189 oestradiol [17], has been reported to prevent telomerase shortening in patients with
190 genetic defects in telomere maintenance and repair [4]. This suggests that aromatization
191 to an estrogen capable of activating an estrogen receptor does not appear to be
192 necessary for the effect of danazol on telomere length [18]. Given that either androgens,
193 oestrogens or both may be involved in the regulation of telomere length, we studied
194 men receiving ADT, as ADT reduces both testosterone and its metabolites DHT and

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195 oestradiol to castrate levels. Moreover, ADT promotes the development of
196 sarcopaenia [10], of structural bone decay [9] and of insulin resistance [11], all features
197 associated with biological aging in men. Given the negative findings of our study, further
198 work is required to determine whether telomere shortening plays a role, if any, in
199 promoting ADT-associated adverse effects resembling that of accelerated aging.
200
201 To date, clinical studies assessing the effects of sex steroids in telomere length have
202 been limited to cross-sectional studies among community dwelling men [5, 19]. This study,
203 to our knowledge, constitutes the first longitudinal controlled study examining the
204 effects of changes in circulating sex steroids on telomere length. Only ADT-naïve men
205 without clinical androgen deficiency who had, prior to commencing ADT, normal
206 circulating testosterone levels were recruited. The focus on men receiving ADT ensured
207 that both testosterone and oestradiol were reduced to castrate levels. In addition, the
208 inclusion of an age- and radiotherapy and comorbidity- matched control group
209 facilitated the delineation of specific effects of ADT. In addition, men were relatively
210 healthy, living in the community without functional impairment receiving adjuvant ADT
211 with curative intent, and all participants maintained undetectable prostate specific
212 antigen levels throughout the study [12].
213
214 The main limitation of the study is its relatively small sample size, especially with
215 regards to the 2 year time point, and hence this study lacks the power to detect modest
216 effects on telomere length. Indeed, this is a secondary analysis of a study powered to
217 detect the effect of ADT on biomechanical leg muscle function [12]. In addition, 2 years is a
218 relatively short time frame, and a longer duration may well be necessary to detect a
219 significant effect of sex steroid deprivation. However, the severe reduction of sex
220 steroid levels following ADT is much more rapid and profound in magnitude than the
221 age-related decline in testosterone levels among community dwelling men not receiving
222 ADT [20]. The study was not powered to assess whether changes in telomere length
223 during ADT are associated with accelerated clinical features of aging observed during
224 ADT, such as loss of muscle mass and function [10, 12], loss of bone mass [9] or metabolically
225 unfavourable changes in body composition[11, 21] .
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227 While we did not use Southern-blot based quantification of telomere length, the qPCR-
228 based methodology used here has been reported to be strongly correlated to Southern-
229 blot methodology with reported correlation coefficients of ranging from r =0.84 to
230 >0.9 [13,22]. To minimize measurement error of LTL over time, care was taken to ensure
231 that all samples were extracted and processed by the same technician using the same
232 protocol in one laboratory.
233 Exposure to significant ionizing radiation has been reported to be inversely correlated
234 with LTL [23]. In the absence of a control group not receiving radiation, we cannot
235 discount possible effects of pelvic irradiation on telomere length. However, given that
236 our objective was to assess the effects of sex steroid deprivation on telomere length,
237 inclusion of a radiotherapy-matched control group should have eliminated possible
238 confounding effects of radiation treatment.
239
240 In conclusion, in this 2-year prospective case controlled study we found no evidence
241 that severe sex steroid deprivation accelerates telomerase shortening in men who have
242 been diagnosed with prostate cancer. However larger, longer term studies are required
243 to confirm, or to refute these findings.
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258 **Declaration of interest:**

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259 There was no conflict of interest that could be perceived as prejudicing the impartiality
260 of the research reported.
261
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266
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270
271 **Author Contribution statement:**
272 A.S. researched data and wrote the manuscript, R.H. researched data, contributed to the
273 discussion and reviewed/edited the manuscript. B.B.Y. researched data and reviewed/
274 edited the manuscript. J.H. researched data and reviewed/ edited the manuscript J.P.B.
275 researched data and reviewed/ edited the manuscript. M.G. designed the study,
276 researched data and wrote the manuscript. A.S. and M.G. are the guarantors for the
277 study.
278
279
280
281
282
283 **Figure Legends**
284 Figure 1. Circulating total testosterone and oestradiol levels in men receiving ADT and
285 matched controls
286 Shown are serum total testosterone (Figure 1a) and oestradiol levels (Figure 1b)
287 (adjusted mean, 95% CI) in men with prostate cancer receiving ADT (continuous line)
288 and aged and radiotherapy matched prostate cancer controls (dashed line).
289 In cases were sex steroids levels were undetectable, levels were set at the lower limit of
290 detection, 0.4 nmol/l for total testosterone, and 19 pmol/l for oestradiol respectively. P
291 values refer to the overall significance of the change between groups during follow-up.

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292

293 Figure 2. Leukocyte telomere length in men receiving ADT and matched controls

294 Shown are leukocyte telomere lengths, expressed as T/S ratio (adjusted mean, 95% CI)
295 in men with prostate cancer receiving ADT (continuous line) and aged and radiotherapy
296 matched prostate cancer controls (dashed line). The P value refers to the overall
297 significance of the change between groups during follow-up.

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389
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396 **Table 1. Baseline characteristics**

397

Baseline Characteristic	ADT group N=40	Control group N=25	p value
Age	67.0 [61.3; 73.3]	70.6 [65; 73.3]	0.21
Prostate Cancer Gleason Score	9 [7; 9]	7 [7; 7]	<0.01
Concurrent radiotherapy treatment	91.2%	88.9%	0.74
Previous prostatectomy	54.3%	77.8%	0.06
Total testosterone (nmol/L)	13.2 [9.2; 18.5]	15.5 [11.1; 17.0]	0.55
PSA (ug/L)	2.0 [0.16; 12.8]	0.07 [0.03; 0.35]	<0.01
Charlson co-morbidity index	2 [2; 3]	3 [2; 3]	0.17
Medical comorbidities			
Ischaemic heart disease	26.5%	14.8%	0.30
Diabetes mellitus	17.6%	18.5%	0.89
Liver disease	0%	0%	>0.99
Chronic kidney disease	0%	0%	>0.99
Hypertension	55.9%	59.3%	0.70
LTL, T/S ratio	1.01 [0.89;1.13]	0.94 [0.86;1.04]	0.106

398

399 PSA = prostate specific antigen; LTL = leukocyte telomere length; T/S = telomeric DNA/
400 single-copy control gene. Data presented are median [interquartile range] or
401 proportions (%). Gleason score <7 = low-moderate risk, 7= intermediate risk, 8-10 =
402 high risk prostate cancer.
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409
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415 **Table 2. Leukocyte Telomere Length**

LTL, T/S ratio	ADT Group	n	Controls	n	Mean adjusted difference [95% CI]	p value
0 months	1.01 [0.89;1.13]	40	0.94 [0.86;1.04]	25		
6 months	1.05 [0.92;1.19]	35	0.95 [0.90;1.10]	25	-0.006 [-0.077;0.069]	
12 months	1.07 [0.96;1.22]	28	1.03 [0.94;1.10]	22	0.003 [-0.072;0.078]	
24 months	1.06 [0.99;1.33]	7	0.99 [0.93;1.11]	12	0.105 [-0.004;0.213]	0.235

416
417
418 Medians [interquartile ranges] are presented. Mean adjusted difference refers to the
419 change over 12 months across groups (mixed model). n denotes the number of study
420 subjects at each time point in each group. The P value refers to the overall significance
421 of the change between groups during follow-up.

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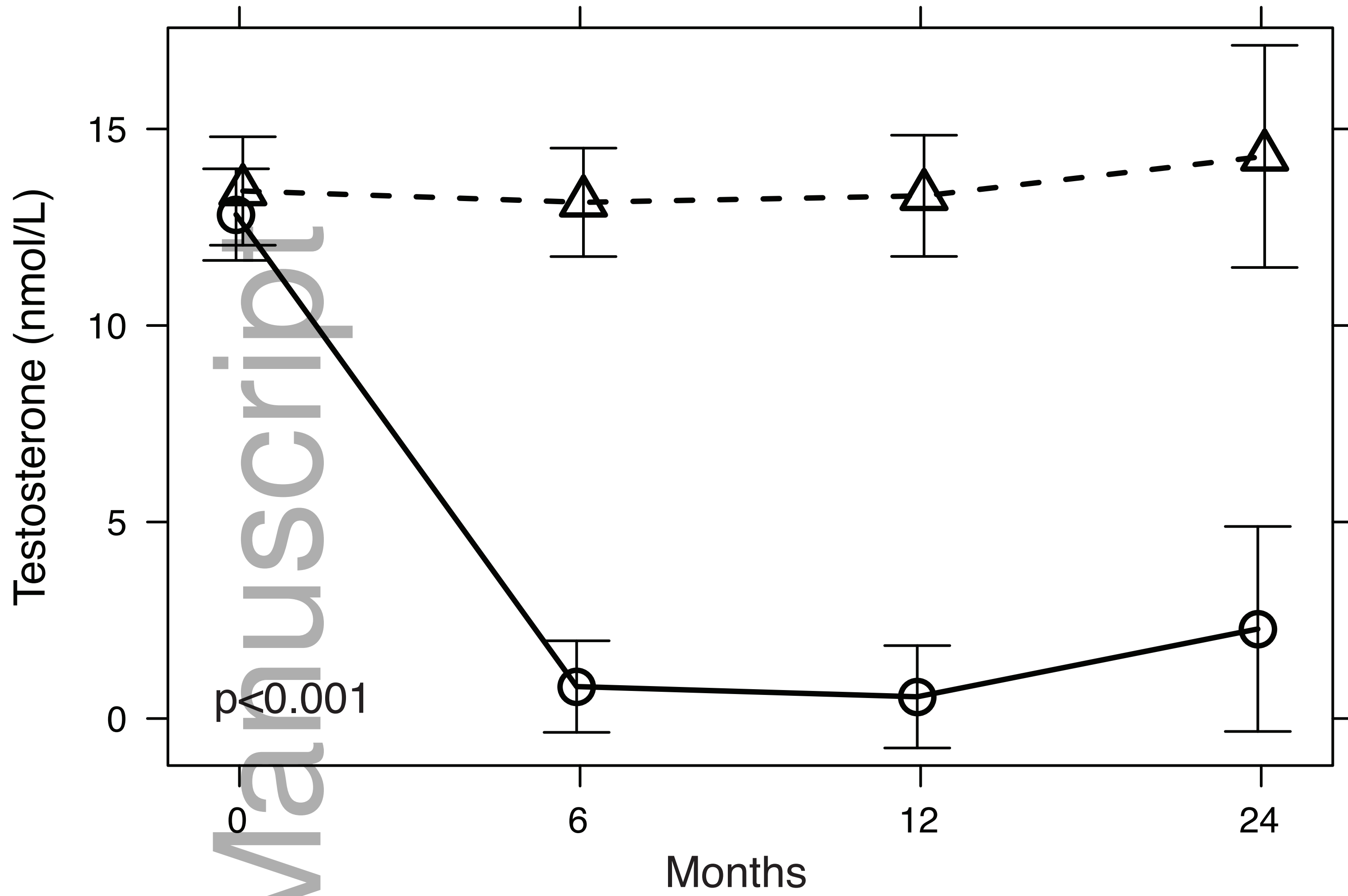

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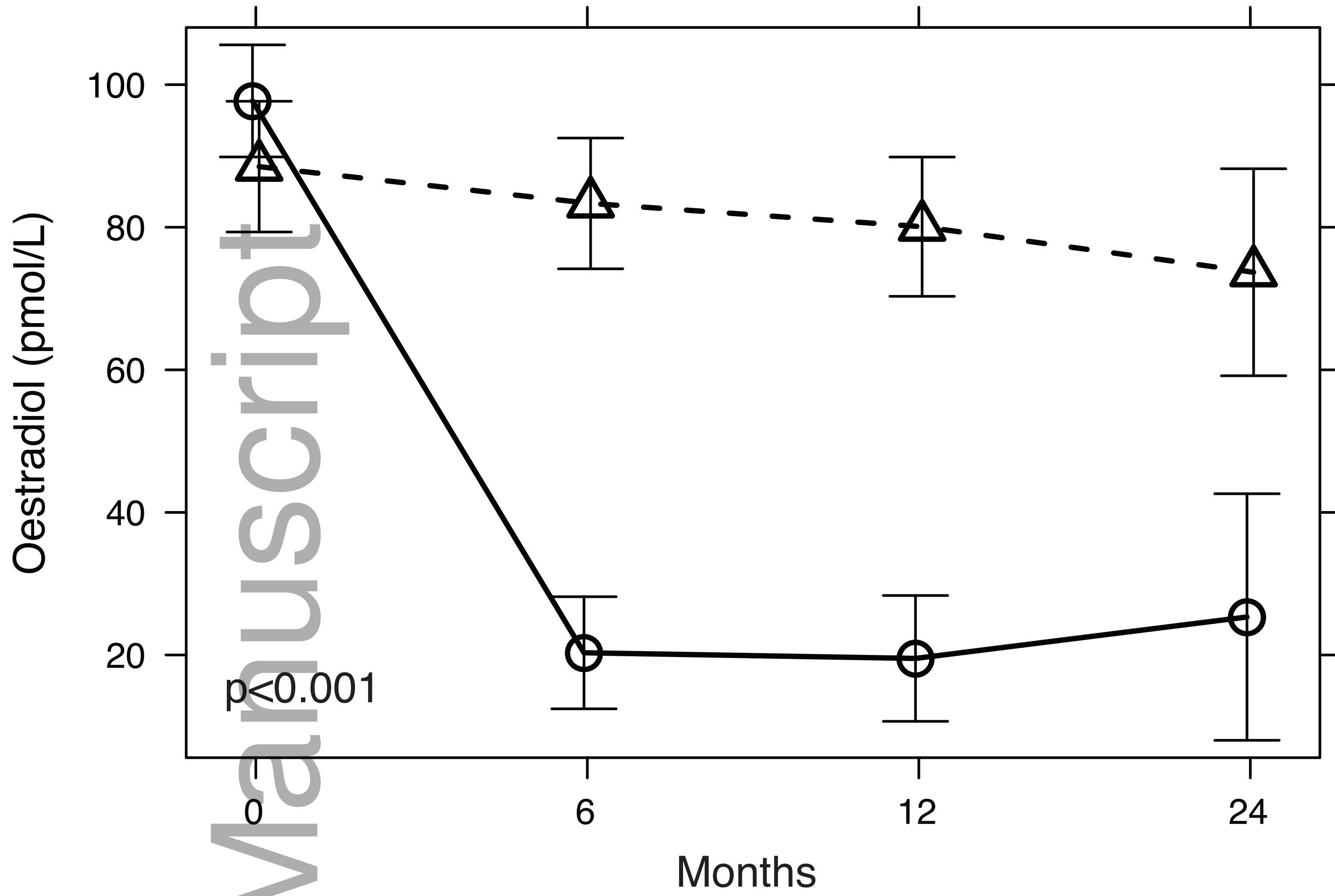

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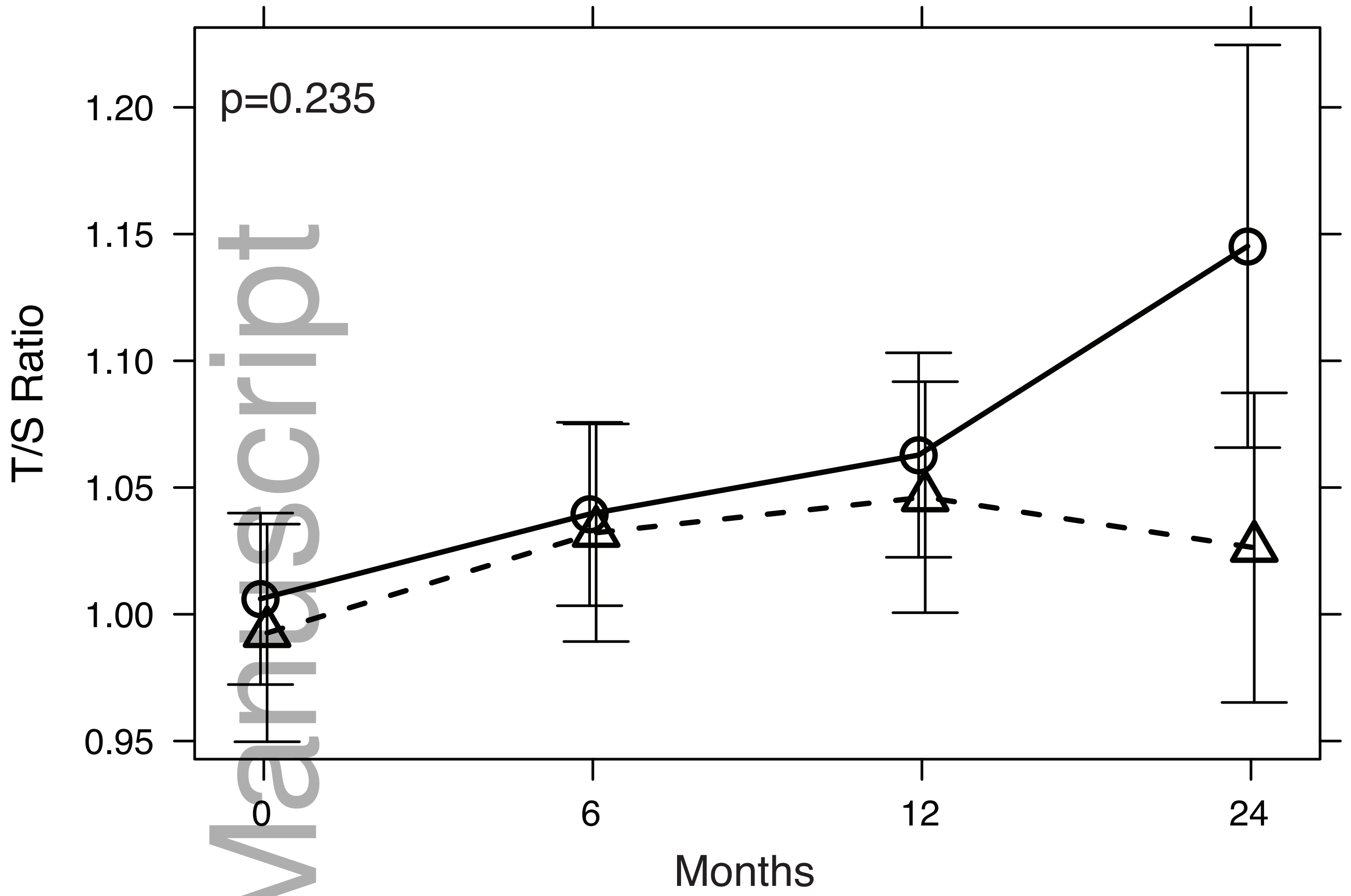

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