A simple method for measuring long-term integrated testosterone levels in men

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24 **Abstract**

25 Steroids play multiple roles in the regulation of development, physiology, reproduction, and
26 behavior. Measuring circulating steroids is especially challenging since concentrations are
27 extremely labile, responding to stressors within minutes. Matrices that integrate long-term
28 steroid levels are therefore valuable as biomarkers of baseline, as well as chronic steroid
29 exposures. Here we report on a simple method to extract and measure accumulated
30 testosterone from human fingernails using commercial EIA kits. Further, we demonstrate
31 known human testosterone sex and age trends. Thus, this method is a potential tool for
32 biomonitoring endogenous as well as exogenous steroid exposure.

33

34 **Key words:** biomonitoring, endogenous steroid levels, integrated matrices, non-invasive,
35 fingernails.

36

37 **Introduction**

38 Steroid hormones influence and are influenced by development, physiology, and behavior.
39 Circulating and integrated steroid levels can provide information on growth and reproduction,
40 and offer prospective biomarkers for well-being and survival (Koren et al. 2012). Measuring
41 steroids in integrated matrices, such as hair, feathers, and nails, is a progressively developing
42 field. Unlike traditional blood sampling, hair and nail collection is relatively non-invasive,
43 pain and infection-free. Hair and nails may be cut and collected quickly and efficiently,
44 without the need for patient privacy or researcher health precautions. They are also easy to
45 transport and store, without needing electricity and cooling. Moreover, while circulating
46 steroid levels represent the momentary total (i.e., protein-bound and free) state, hair and nails
47 provide a longer time-frame, presumably integrating free steroids over the time of their
48 growth (i.e., weeks or months).

49 In mammals, nails develop by keratinocytes cell divisions in the proximal germinal
50 regions (Baran 1981; De Berker et al. 2007). Nail growth is attributed primarily to the nail
51 matrix and to a lesser extent to the nail bed. The nail matrix is mainly composed of specific
52 keratin proteins, cross-linked by keratin-associated proteins. Diverse factors influence nail
53 growth, including numerous physiological and pathological conditions (reviewed in Zaiac &
54 Walker (Zaiac & Walker 2013)). On average, human fingernails grow at a rate of 3 mm per
55 month, while human toenails grow at a rate of 1 mm per month. During nail growth, multiple
56 materials are transferred from the capillary blood vessels nourishing the nail to the growing
57 nail (Palmeri et al. 2000). These are embedded in the nails, making this abundant matrix an
58 attractive candidate for biomonitoring multiple blood borne substances. Indeed, poisonous
59 materials (Barbosa et al. 2005; Button et al. 2009; Mehra & Juneja 2005; Suzuki et al. 1989),
60 drugs (Shu et al. 2015) and endocrine-disruptors (Li et al. 2013) have been forensically
61 detected in human nails. In addition, endogenous steroids have been analyzed in nail extracts.
62 For example, infants that experienced in utero stress had higher DHEA
63 (dehydroepiandrosterone) compared to infants from non-stressed mothers (Tegethoff et al.
64 2011). In another study, nail cortisol-to-DHEA ratio was found to be related to perceived
65 exam stress in students (Warnock et al. 2010). Exogenous testosterone and its derivatives
66 were measured in nails using LC-MS (Brown & Perrett 2011). However, the authors
67 concluded that detection of these substances was not sensitive enough. GC-MS was used in a
68 different study, yet this method requires derivatization (Choi et al. 2001). Here, we aimed to
69 develop a simple and repeatable quantitative method for measuring endogenous testosterone
70 in nails, and biologically validate it using known age-related trends.

71

72 **Materials and methods**

73 *Nails collection*

74 The study was approved by Bar Ilan University's Research on Human Subjects' Ethics
75 Committee. In order to collect nail samples, we sent an electronic mail to all graduate

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76 students, staff, and faculty members in the Faculty of Life Sciences at Bar Ilan University,
77 requesting fingernail donations from men, women and children. Samples were placed in an
78 empty paper envelope marking only the sex and age of the donator on it. Envelopes were
79 deposited anonymously in our laboratory mailbox. Between November 2014 and May 2015
80 we collected 52 samples from men (ages 18.5-74), 6 samples from boys (ages 0.4-2), 11
81 samples from women (ages 18-53) and 11 samples from girls (ages 3-9).
82

83 *Steroid extraction*

84 We extracted steroids from nails using our published protocol for hair and claw-testing
85 (Koren & Geffen 2009; Koren et al. 2006; Koren et al. 2008; Koren et al. 2002; Koren et al.
86 2012; Matas et al. 2016), with several modifications. Nails were weighed to the nearest 0.01
87 mg in safe-lock polypropylene tubes (Sarstedt, Germany). Samples were ground in mixer mill
88 MM 400 (Retsch, Germany) for 10 minutes at 25 Hz. Methanol (UPLC grade, Sigma, Israel)
89 was added, and the minced samples were sonicated for 30 minutes and then incubated
90 overnight at 50°C, with gentle shaking. Next day, the supernatant was centrifuged at 17,000 g,
91 transferred into a glass tube (Corning Inc., USA) and evaporated under a stream of nitrogen.
92 Samples were reconstituted and testosterone was quantified in the assay buffer that was
93 provided with the commercial enzyme-linked immunosorbent assays (ELISA) according to
94 manufacturer's recommendations.
95

96 *Quantitation of steroids*

97 High sensitivity salivary ELISA kits (Salimetrics; item no.1-2402; Ann Arbor, USA) were
98 validated for nails by examining parallelism with kit standards and linearity. Serial dilutions
99 of separate pools for men, women and children all showed parallelism with the provided kit
100 standards (univariate analysis of variance in SPSS; P=0.5; P=0.46 and P=0.48 for men,
101 women, and children respectively). Linearity was demonstrated for men between 1-20 mg of
102 nail extract, corresponding to 6-230 pg/mL of kit standard. For women, linearity was

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103 observed in the 30-50 mg nail extract range, corresponding to 100-200 pg/mL standards. In
104 children, serial dilutions were linear in the 10-50 mg nail extract range, which is equivalent to
105 38-210 pg/mL salivary standards. The lowest concentration that we detected from a single
106 individual using the assay was 0.7 pg/mL, corresponding to 3.95 mg of nail matrix. According
107 to the manufacturer, antibody cross-reactivity was reported as 36.4% with
108 dihydrotestosterone, 21.02% with 19-nortestosterone, 1.9% with 11-hydroxytestosterone,
109 1.157% with androstenedione and less than 0.489% for all other steroids. By measuring six
110 samples of the pool on the same ELISA plate we determined intra-assay variability to be
111 2.7%. By quantifying four samples of the pool on four different days we calculated inter-
112 assay variability to be 11.78%. Recovery was calculated to be 86% by the addition of a
113 known amount of testosterone to the nail extract. The presence of testosterone in human nails
114 was further validated utilizing a second antibody, by the DRG testosterone ELISA kit (DRG
115 International item no. EIA-1559; NJ, USA) following the manufacturer's protocol. DRG
116 reports that the testosterone antibody cross-reacts with 11β-Hydroxyestosterone (3.3%), 19-
117 Nortestosterone (3.3%) and other steroids (≤0.9%).
118

119 **Results and Discussion**

120 We developed a simple protocol for human fingernail testosterone extraction and quantitation.
121 According to our validations, whereas 5 mg of nails were needed to quantify testosterone in
122 men, ten-fold was necessary to quantify testosterone in women and girls (i.e., 50 mg). This is
123 comparable to the documented association between circulating testosterone in men vs. women
124 (Longcope, 1986). Since a large amount of nail matrix was needed for the female analysis, we
125 created a pool from all donated female nails. Figure 1 shows nail testosterone in adult men
126 (mean for N=52; 5.04 pg/mg nails), women (mean for N=11; pool; 2.3 pg/mg nails), boys
127 (mean for N=6; 1.8 pg/mg nails), and girls (mean for N=11; pool; 1.07 pg/mg nails). In order
128 to perform statistical comparisons, testosterone levels were transformed using the Johnson Su
129 transformation for normal distribution. All analysis was performed in JMP 12 (SAS Institute
130 Inc.). Analysis of variance showed significant differences in testosterone levels between sex

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131 and age groups ($F_{59,3}$=9.665; P<0.0001). Tukey-Kramer post-hoc statistics (q=2.648; α=0.05)
132 showed significant differences in nail testosterone levels between men and boys (difference
133 1.758; P<0.0001). These results support previous findings reported in the literature where
134 testosterone concentrations are higher in men and women than in boys and girls (Andersson et
135 al. 1997; Mouritsen et al. 2014). In this analysis, we found a two-fold difference between
136 men and women's testosterone levels. Established normal ranges of circulating testosterone
137 concentrations indicate a five-fold difference between low levels in men (usually 300 ng/dL)
138 and high levels in women (usually 60 ng/dL). This discrepancy may be attributed to sampling
139 size, age and seasonal fluctuations, as well as specific matrix effect. Whereas blood samples
140 reflect a momentary concentration, nails show accumulated long-term levels, which integrate
141 concentrations over time.

142 We found a significant association between age and testosterone in male nails
143 (R^2=0.405; N=58; P<0.0001; Figure 2). As documented in the literature (Vermeulen et al.
144 1972), testosterone levels reached a maximum in men in their 20s and than gradually
145 decreased. Although the variation in circulating testosterone is high, the pattern is profound.
146 In this study, we use commercial EIA kits to quantify testosterone. Immunoassays are widely
147 used to quantify steroids due to their high sensitivity and procedural simplicity. Although
148 mass spectrometry is superior in terms of specificity and flexibility to measure multiple
149 compounds simultaneously, a large amount of tissue is initially needed, due to loss during
150 sample prep and cleanup. For example, Choi *et al*. (2001) used 100 mg of nail matrix to find
151 differences between testosterone in men and women using GC-MS(Brown & Perrett 2011;
152 Choi et al. 2001), and Brown and Perrett (2011) did not find differences between males and
153 females or exogenous testosterone users and non-users (Brown & Perrett 2011). Here we
154 present a simple and reliable antibody-based method for testosterone quantitation using a
155 minimal amount of nail matrix, without the need of derivatisation or costly equipment.
156 Testosterone has been implicated in various diseases, fertility issues, anxiety and stress-
157 related disorders, as well as risk-taking, sexual, and aggressive behaviors. Nails may serve as
158 an ideal matrix for long-term measurements of baseline or chronic testosterone levels related

159 to stable physiological or psychological states, behavioral syndromes, and personality traits,
160 as well as follow-up of exogenous testosterone exposure. Nail collection is relatively non-
161 invasive, and samples are easy to store and safe to handle. Other advantages include their
162 bountiful quantity and slow growth, reflecting long-term steroid concentrations. Our method
163 offers the possibility for researchers to quantify steroids in nails in an easy, applicable way,
164 and document stable trends. Utilizing nails for steroid analysis also enables periodic
165 quantitation by repeated cutting, opening an exciting array of questions and comparative
166 studies biomonitoring individual differences in endogenous steroid levels, as well as exposure
167 to exogenous steroids, endocrine disruptors and other environmental pollutants that may
168 interfere with the hypothalamus-pituitary-gonadal pathways.
169
170 **Conclusions**
171 Measuring integrated steroid levels that reflect long-term baseline is highly relevant, since
172 steroid concentrations are labile. Here we present a protocol for extraction and quantitation of
173 testosterone in human fingernails. We biologically validated our methodology by
174 demonstrating known changes in testosterone levels throughout men's lives, and expected sex
175 differences. These allow the usage of this protocol for further comparative research and
176 biomonitoring.
177
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182

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243

244 **Figure legend**

245

246 Figure 1: Nail testosterone levels in men, women and children. Testosterone was extracted
247 from 5 mg of nail clippings of individual men (N=52) and boys (N=6). Error bars were
248 constructed using 1 standard error from the mean. For women (N=11) and girls (N=11)
249 testosterone was extracted from 50mg of pooled nails. Significant differences were seen
250 between sex and age classes (ANOVA: $F_{59,3}=9.665$; $P<0.0001$).

251

252 Figure 2: Transformed nail testosterone as a function of male age. Transformed testosterone =
253 $-0.151 + 0.0245*age - 0.0016*(age-28.06)^2$. Johnson Su transformation was used to
254 normalize the distribution of testosterone. Polynomial second degree fit $R^2=0.405$;
255 N=58; $P<0.0001$

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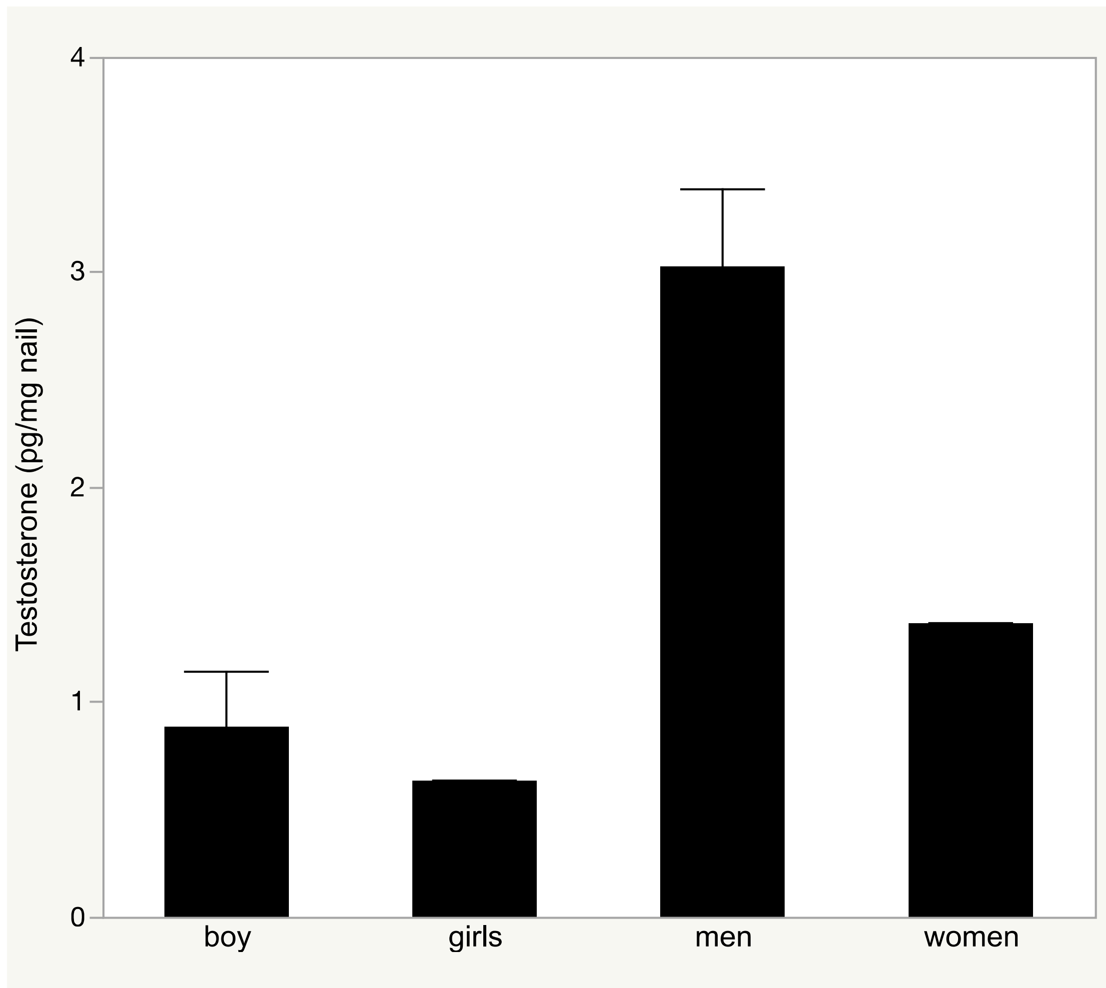
256 Figure 1

257



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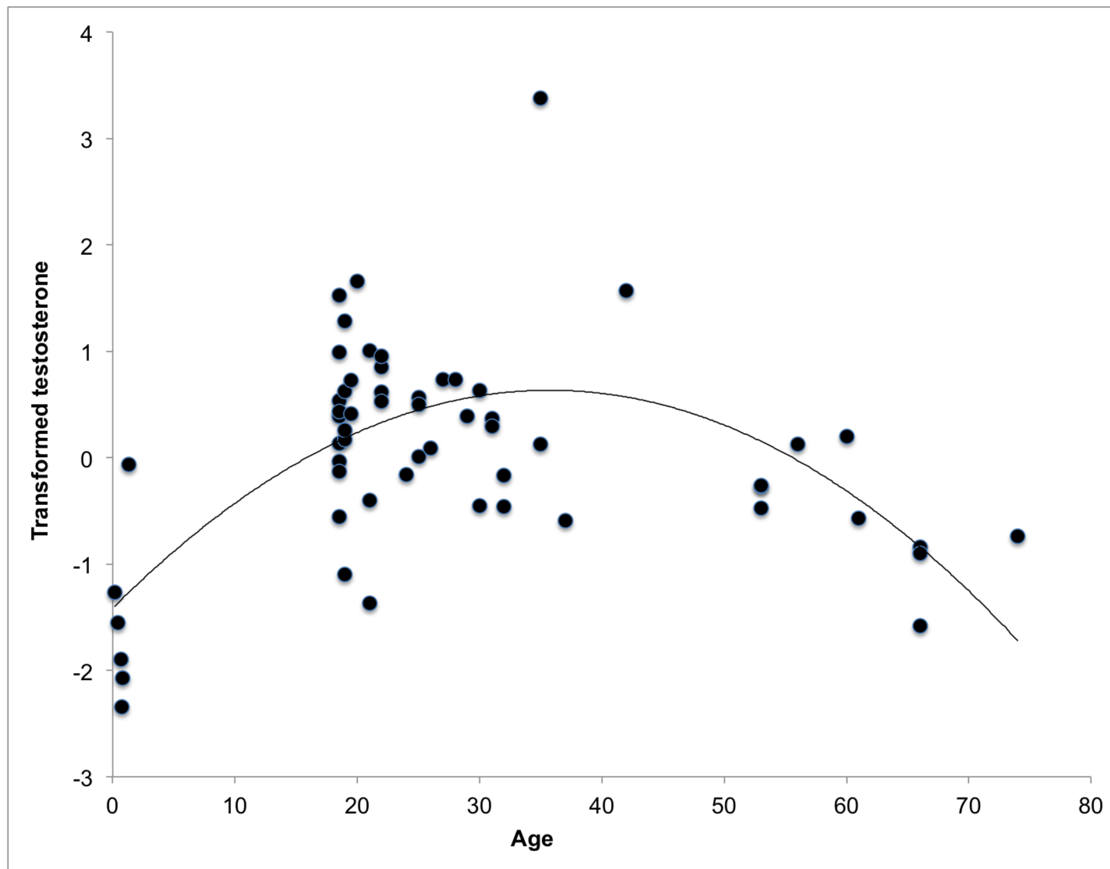
260 Figure 2



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