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8 **Testicular growth and spermatogenesis: new goals for pubertal hormone replacement in boys**
9 **with hypogonadotropic hypogonadism?**

10 **A multicentre prospective study of hCG/rFSH treatment outcomes during adolescence**

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34
35 **Abbreviated title:**

36 **Gonadotrophin replacement in boys with hypogonadotropic hypogonadism**

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ABSTRACT

Context/objective: Testosterone treatment for pubertal induction in boys with hypogonadotropic hypogonadism (HH) provides virilisation, but does not induce testicular growth or fertility. Larger studies evaluating the outcomes of gonadotrophin replacement during adolescence have not been reported to date; whether previous testosterone substitution affects testicular responses is unresolved. We aimed to assess the effects of human chorionic gonadotrophin (hCG) and recombinant FSH (rFSH) in boys and adolescents with HH with respect to a) testicular growth, b) spermatogenesis, c) quality of life (QoL) and to identify factors influencing therapeutic success.

Design/setting: A prospective case study was conducted in 26 paediatric endocrine centres

Patients/interventions: HCG and rFSH were administered until cessation of testicular growth and plateauing of spermatogenesis to (1) pre-pubertal HH boys with absent or early arrested puberty (group A) and to (2) HH adolescents who had previously received full testosterone replacement (group B). **Outcome measures:** bi-testicular volumes (BTVs), sperm concentrations and QoL.

Results: Sixty (34A/26B) HH patients aged 14-22years were enrolled. BTVs rose from 5 ± 5 to 34 ± 3 ml in group A vs. 5 ± 3 to 32 ± 3 ml in group B, with normal final BTVs (≥ 24 ml) attained in 74%/70% after 25/23 months in A/B respectively. Sperm in the ejaculate were found in 21/23(91%)/18/19(95%), with plateauing concentrations after 31/30 months of hCG and 25/25 months of combined treatment. Sperm concentrations were normal (≥ 15 mill/ml) in 61%/32%, with mean concentrations of 40 ± 73 vs. 19 ± 38 mill/ml in A/B (n.s.). Outcomes were better in patients without bilateral cryptorchidism, with non-congenital HH causes, higher baseline BTVs, and higher inhibin B and AMH levels. QoL increased in both groups.

Conclusions: HCG/rFSH replacement during adolescence successfully induces testicular growth and spermatogenesis, irrespective of previous testosterone replacement, and enhances QoL.

75 **Introduction**

76 In boys ≥ 14 years with hypogonadotrophic hypogonadism (HH) and absent or arrested puberty,
77 pubertal induction is performed by administering increasing doses of testosterone-enanthate i.m every
78 3-4 weeks. This regimen, established as a therapeutic standard in paediatric endocrinology, stimulates
79 normal linear growth, pubertal virilisation and psycho-sexual maturation, but neglects testicular
80 growth and the acquisition of fertility as components of normal puberty; the testes remain in an
81 immature pre-pubertal state (i.e. < 4 ml), and spermatogenesis is not initiated. Replacement of
82 gonadotrophins in adulthood has repeatedly been proven to be safe and effective in initiating testicular
83 growth and spermatogenesis, sufficient for fertility ^{1, 2, 3, 4, 5}. Small adolescent case studies have
84 demonstrated the "proof of principle" that, along with pubertal virilisation, pubertal testicular
85 maturation with increase in testis sizes and initiation of spermatogenesis can be achieved by combined
86 hCG/FSH replacement ^{6, 7, 8, 9, 10, 11}. However, prospective studies in HH adolescents large enough for
87 evaluation of outcomes of hCG and FSH have not been reported to date. Although a recent paper¹²
88 specifically addressed quality of life (QoL) in relation to gonadotrophin treatment for adult HH, the
89 impact of gonadotrophin substitution on QoL during adolescence is largely unknown. Whether
90 preceding testosterone replacement may adversely affect therapeutic responses of HH adolescents also
91 remains unresolved to date.

92 In this prospective multicentre study we aimed to assess the effects of human chorionic gonadotrophin
93 (hCG) and recombinant FSH (rFSH) treatment in young patients with HH of various origins with
94 respect to testicular growth and induction of spermatogenesis. We compared the outcomes of pre-
95 pubertal HH boys with those of HH adolescents who had previously received full testosterone
96 replacement for pubertal virilisation. Furthermore, we evaluated pre- and post-treatment QoL in each
97 patient using validated questionnaires. Finally, we assessed the dependence of therapeutic response on
98 variables at baseline.

99

100 **Patients and methods**

101 The study was performed over 4 years, between 3/2011 and 3/2015, in 26 centres for paediatric
102 endocrinology throughout Germany and coordinated by the first author at the Department for Clinical
103 Andrology, Centre for Reproductive Medicine, University of Münster/Germany.

104

105 **Ethics**

106 Informed written consent by majors, and assent by minors with consent of their parents was obtained
107 for all procedures. The study was approved by the Ethics Committee of the State Medical Board of
108 Westfalen-Lippe (approval number: 2010-427-f-S).

109

110 **Inclusion criteria**

111 Males aged 14-22 years with hypogonadotrophic hypogonadism (HH) were enrolled in the study.
112 Subsets of participants were as follows: Boys/adolescents with:

113 -absent puberty by age 14, confirmed by testicular volumes <4 ml each side, pre-pubertal levels of LH,
114 FSH and testosterone and failure of GnRH agonist (buserelin, 10 µg/kg s.c) to stimulate LH >4 U/l
115 after 4 hours and/or absent pubertal response to “priming” with low-dose (50-100mg) testosterone-
116 enanthate i.m. over 3-6 months.

117 -early pubertal arrest after age 14, confirmed by arrested testicular growth (with volumes >4≤8 ml
118 each side) and pre-pubertal levels of LH, FSH and testosterone.

119 -Kallmann syndrome, confirmed by presence of anosmia or severe hyposmia (by “Sniffin-sticks”,
120 Burghart Messtechnik GmbH, Wedel, Germany) or *KAL1* mutation.

121 -congenital or acquired multiple pituitary hormone deficiencies (MPHDs)

122 -CHARGE syndrome.

123

124 ***Exclusion criteria***

125 Patients with constitutional delay of growth and puberty (CDGP), testicular disorders (primary
126 hypogonadism), Prader-Willi syndrome, functional hypogonadism (due to eating disorders or chronic
127 diseases) or with HH due to untreated abnormalities of the hypothalamic-pituitary region were
128 excluded.

129

130 ***Primary study end points***

131 -Final bi-testicular volumes (BTVs= the sum of both testicular volumes) by Prader orchidometry

132 -Final sperm concentrations (SCs), according to WHO 2010 criteria¹³

133

134 ***Secondary study endpoints***

135 -QoL post-gonadotrophin treatment, compared to pre-treatment. Assessed by three standardised
136 questionnaires: “Inventory for assessment of quality of life in children and adolescents” (“ILK”)¹⁴,
137 “Inventory for assessment of depression in children and adolescents” (“DIKJ”)¹⁵, and “Questionnaire
138 for assessment of emotion regulation strategies in children and adolescents” (“FEEL-KJ”)¹⁶, and
139 by two questions on self-perceived satisfaction with testis size and masculinity

140

141 ***Treatment protocols***

142 Patients were divided into two groups:

143 **Group A** included pre- pubertal HH boys (Tanner stage G1 with testicular volumes (TVs) <4 ml each
144 side or HH boys with early pubertal arrest (Tanner G2-3 with TVs >4≤8 ml).

145 **Group B** comprised fully virilised (Tanner G4-5, TVs <4ml each side) HH adolescents who had
146 received at least 1.5 years of full (250 mg i.m. every 3-4 weeks) testosterone-enantate replacement.

147

148 Highly purified urinary-derived hCG (Brevactid®), followed by combined hCG/rFSH (Gonal f®) was
149 self-administered with or without parental help via subcutaneous injections, two injections/week (on

150 Mondays and Fridays) for hCG and three injections/week for rFSH (on Mondays, Wednesdays and
151 Fridays).

152 The end of gonadotrophin replacement was defined by cessation of testicular growth and plateauing of
153 sperm concentrations in the ejaculate over at least two observations at 3 monthly intervals.

154

155 **Protocol group A:** For testosterone-naïve pre-pubertal boys (or early pubertal boys with arrested
156 puberty), the following gonadotrophin replacement protocol was recommended: As pre-
157 pubertal boys have not yet attained their adult height, a relatively low starting dose of (250-)
158 500 IU hCG was injected subcutaneously on Mondays and Fridays, and incremental increases
159 of 250-500 IU hCG per injection every 6 months to a maximum of 3 x 2500 IU hCG
160 s.c./week were recommended. The aim was to achieve pubertal levels (serum testosterone
161 ≥ 1.5 ng/ml, [5.2 nmol/l]) after around 6 months, and levels in the mid-normal adult range
162 (testosterone >3.5 ng/ml, [12 nmol/l]) by one year. rFSH (follitropin alpha) 3 x (75-)150
163 IUs.c/week (injected Mondays, Wednesdays and Fridays) was added when pubertal serum
164 testosterone levels (>5.2 nmol/l) were reached, without subsequent rFSH dosage
165 modifications above 150 IU per injection, aiming at physiologic serum FSH target levels between
166 1-7 U/L.

167 **Protocol group B:**

168 In those adolescents previously treated with testosterone who had completed pubertal virilisation
169 and linear growth (documented by left hand digital epiphyseal fusion), the following gonadotrophin
170 replacement protocol was used: A full (adult) hCG starting dose of 1.500 IU s.c. was initially
171 applied twice weekly (injected subcutaneously on Mondays and Fridays). HCG dose
172 reduction was recommended if erythrocytosis, gynaecomastia or excessive acne occurred. If
173 testosterone levels remained below the normal adult range (<12 nmol/l) after 6-9 months, the
174 hCG dose was increased by increments of 500(-1000) IU per injection every 6 months until
175 achievement of serum testosterone levels ≥ 12 nmol/l, but not above a maximum of 3 x 2500
176 IU hCG s.c./week. In all patients rFSH (follitropin alpha) 150 IU was additionally injected
177 thrice weekly (on Mondays, Wednesdays and Fridays) after 3 months of hCG, without
178 subsequent dose modifications, aiming at physiologic serum FSH target levels between 1-7 U/L.

179 **Baseline, follow-up, and outcome measurements**

180 All boys were examined at baseline and during three-monthly follow-up visits while undergoing
181 gonadotrophin substitution; annual bone age estimations were performed in group A.

182 Individual clinical details at baseline (including underlying causes of HH, and presence or absence of
183 previous cryptorchidism) and outcome data (primary study endpoints) are described in Table 1.
184 Testicular volumes were measured clinically using a Prader orchidometer; summated left plus right

185 testicular volumes (BTVs) were calculated. To confirm testicular growth and to rule out intra-
186 testicular pathologies, ultrasound investigations were additionally performed (using the formula:
187 length x width x depth/2 for baseline and follow-up volumes and the ellipsoid method¹⁷ for final
188 volumes). AMH and inhibin B levels were assessed at baseline, at initiation of rFSH replacement and
189 on final assessment. LH levels were measured to rule out spontaneous activation of the hypothalamic-
190 pituitary-gonadal (HPG) axis on gonadotrophin substitution; serum testosterone levels were measured
191 every three months to monitor Leydig cell response and, along with FSH levels, adherence to
192 treatment. Once psycho-sexual maturity was attained, ejaculates were collected (by masturbation)
193 after at least 48h of sexual abstinence, and thereafter repeated every three months until plateauing of
194 sperm concentrations in the ejaculate was documented in two follow-up visits. All samples were
195 analysed for volume, sperm concentrations, progressive motility and morphology¹³; total sperm counts
196 were calculated. By the end of gonadotrophin substitution, all patients able to provide a semen sample
197 had a final centralised assessment by one experienced physician (JR), comprising primary and
198 secondary endpoints and final height.

199

200 ***Laboratory methods***

201 Inhibin B and AMH levels were analysed in frozen blood serum samples in the central study centre:
202 Inhibin B (solid phase sandwich assays, Beckman-Coulter; intra-assay coefficient of variation (CV):
203 3.3%; (high control: 4.9%); detection limit (DL): 10 pg/ml. Cross-reactivity with inhibin A: 1%).
204 AMH (ELISA, DRG Instruments GmbH; CV: 5.7% (high control: 9.0%); DL: 0.14 pg/ml).
205 The other standard hormone investigations (LH, FSH, testosterone) were performed by the
206 participating centres during hCG/rFSH substitution and again in the study centre at final assessment.

207

208 ***QoL assessment***

209 All patients were asked to fill in four questionnaires at baseline and by the end of gonadotrophin
210 replacement. The ILK questionnaire included 9 rating items on the adolescent's perception of his
211 situation in life, involving school, family, personal interests and leisure activities, physical fitness,
212 mental fitness, disease burden and burden by therapeutic interventions. Evaluation was performed by
213 calculation of health-related QoL scores (normal: 70-100%) and problem scores (on severity scale
214 from 1-7). The DIKJ questionnaire included 26 items on the adolescent's emotional and somatic state,
215 including negative feelings and consequences of depressive mood. Evaluation was performed by
216 calculation of t-scores (with significant depression defined as a score >60%). The FEEL-KJ
217 questionnaire included 24 items assessing adaptive and maladaptive strategies for emotional
218 regulation. Two additional questions evaluated "satisfaction with testis size" and "satisfaction with
219 masculinity" on a scale from -2 to +2.

220

221 ***Statistics***

222 Analysis and drafting of figures was performed using Graph Pad Prism 5.0 (GraphPad Software Inc.
223 La Jolla, USA). All results are expressed as the mean \pm SD and additional median (range) for QoL
224 data. Where normality of distribution was determined, t-tests for independent samples were conducted;
225 otherwise the Mann-Whitney-U test was used. Dependence between two variables was assessed using
226 Spearman's rank correlation coefficient. Significance was defined as p-value <0.05 .

227 228 **Results**

229 A total of sixty patients aged 14-22 years with HH were enrolled. Group A boys (n=34), mean age
230 15.5 years, were pre-pubertal or had early arrested puberty. Group B adolescents (n=26), mean age
231 18.8 years, had received previous full testosterone-enanthate replacement for 1.5-5.7 years (mean: 2.5
232 years).

233 Following monotherapy with low doses of hCG replacement, two (additional) previously pre-pubertal
234 patients were recognised as having CDGP and not HH: rising LH serum levels and pubertal testicular
235 growth were observed in these subjects. These patients were not included in the study and hCG
236 replacement was ceased.

237 In group A, three patients discontinued hCG/rFSH replacement; one patient with congenital multiple
238 pituitary hormone deficiencies (MPHD), and 2 patients with congenital normosmic HH (CHH). Four
239 patients had not yet reached the therapeutic endpoints at the time of evaluation of the study, leaving 27
240 participants in group A. Twenty-three group A boys provided semen samples for final assessment. In
241 group B, three patients withdrew from the study: one patient with MPHD after tumour surgery, one
242 patient with congenital MPHD and one patient with CHH, leaving 23 group B participants. Nineteen
243 group B adolescents provided semen samples for final assessment.

244 245 ***Puberty induction***

246 In all group A boys, pubertal virilisation to Tanner stage V occurred without major adverse side-
247 effects. Mild gynaecomastia (Tanner B2-3) was observed in four subjects, severe acne did not occur.
248 Pubertal growth from a mean pre-treatment height of 168 ± 10 cm to a mean final height of 181 ± 8 cm,
249 appropriate for mid-parental target height (180 ± 6 cm) was documented. Bone age matured from
250 14 ± 1.4 to 17 years. Adolescents in group B grew from 176 ± 9 cm (bone age pre-treatment: 16.7 ± 0.7
251 years) to 178 ± 8 cm (parental target: 177 ± 4 cm). Pubertal T levels were reached after 6 ± 3 months of
252 hCG treatment in group A and after 4 ± 3 months in group B.

253 254 ***Endpoints***

255 ***Final bi-testicular volumes (BTVs)*** (Figure 1)

256 Gonadotrophins were administered for 24 ± 7 / 22 ± 6 months in group A/B, respectively until cessation
257 of testicular growth.

258 BTVs rose from 5 ± 5 ml at baseline to 10 ± 8 ml on hCG alone and to 34 ± 3 ml after combined treatment
259 with hCG and rFSH in group A and from 5 ± 3 to 8 ± 5 to 32 ± 3 ml in group B (Figure 1). Changes in

260 testicular sizes in response to gonadotrophin treatment in the different HH patient subsets of both
261 groups (A/B) are detailed in Table 1.

262

263 ***Sperm concentrations (SCs) and other semen parameters***

264 Sperm were found in 91% (21/23) of group A vs. 95% (18/19) of group B patients. Two group A
265 patients (one with KS and one with CHH, both with initial BTVs of 6ml) and one group B patient
266 (with CHH, with initial BTVs of 2 ml) remained azoospermic. Only one of them had a history of
267 bilateral cryptorchidism. Successful microscopic testicular sperm extraction (mTESE) was performed
268 in the latter patient with KS, and mTESE samples were cryostored for potential future use in assisted
269 reproduction. The other two azoospermic patients did not wish to undergo surgery for sperm retrieval.
270 SCs plateaued after 31 ± 6 / 30 ± 7 months from start with hCG and after 25 ± 9 / 25 ± 9 months of
271 combined hCG / rFSH treatment, in A/B, respectively (Figure 2). Final SCs were normal (≥ 15 mill/ml)
272 in 61% (14/23) in group A and 32% (6/19) in group B, and mean SCs were non-significantly higher in
273 A (40 ± 73 mill/ml) than in B (19 ± 38 mill/ml; $p=0.07$).

274 In group B first sperm were found 15 ± 7 months after start of hCG administration and 11 ± 6 months
275 after initiation of FSH treatment. The previously pre-pubertal boys required 2.0 ± 1 years of
276 gonadotrophin replacement before “feeling mature enough” to provide a semen sample for laboratory
277 analysis. In this group, first sperm were documented after 21 ± 10 months of hCG and 17 ± 7 months of
278 combined hCG/rFSH administration.

279 Mean final ejaculate volume was slightly lower than the WHO normal value in group A (A: 1.3 ± 0.2
280 ml; B: 3.8 ± 0.8 ml; normal: ≥ 1.5 ml). Final total sperm counts were not significantly different (A:
281 60 ± 160 mill; B: 42 ± 55 mill; normal: ≥ 39 mill; $p=0.43$), neither was progressive motility (A: $43\pm 18\%$;
282 B: $42\pm 14\%$; normal: $\geq 32\%$), nor sperm morphology (A: $4\pm 3\%$; B: $3\pm 2\%$; normal: $\geq 4\%$).

283

284 ***Quality of life (QoL) before and after gonadotrophin replacement***

285 At baseline, health-related QoL scores (ILK) (Figure 4) were at the lower limit of the normal range in
286 both groups (median: A: 74%; B: 75%). Health-related problem scores (ILK) (on a scale from 1-7)
287 were comparable in A and B, but showed large intra-individual variations (median (range): A: 2.0 (0-
288 7); B: 1.0 (0-5)). Group B adolescents had significantly higher baseline depression scores (DIIKJ)
289 than group A boys, with less variation (median (range): A: 34 (0-100); B: 50 (35-73); A/B pre-
290 treatment $p=0.03$).

291

292 After gonadotrophin treatment, QoL scores were significantly higher than pre-treatment in both groups
293 (A: 86%; pre/post $p=0.03$; B: 82%; pre/post $p=0.03$), accompanied by significantly lower post-
294 treatment problem scores (A: 0; pre/post $p=0.04$; B: 0; pre/post $p=0.01$) and lower depression scores in
295 group A (A: 19 (3-94); pre/post $p=0.05$), while depression scores in group B had not significantly
296 changed (B: 43 (35-66)). When comparing post-treatment scores between the two groups, depression

297 scores of group B were significantly higher than those of A (post-treatment A/B $p < 0.01$), while post-
298 treatment QOL and problem scores were not different.

299 There were no significant changes in response to gonadotrophin treatment in both groups with respect
300 to scores for adaptive and maladaptive strategies of emotional regulation (FEEL-KJ) (data not shown).
301 Self-reported satisfaction (on a scale from -2 to +2) concerning testis size was considerably higher in
302 both groups after gonadotrophin treatment (mean pre-treatment scores A/B: -1.2/-1.2 vs. +1.3/+1.1
303 post treatment in A/B, respectively). Satisfaction concerning masculinity increased more in group A
304 (A: from -0.5 to +0.9 vs. B: from -0.2 to +0.2) (supplementary Figure1).

305

306 ***Analysis of baseline variables potentially influencing therapeutic response to gonadotrophins***

307 ***-Causes of HH***

308 With respect to adherence to treatment, which was better in group A (supplementary Figure 2), there
309 was a trend towards higher final BTVs and higher final sperm concentrations achieved by patients
310 with childhood-acquired causes of HH (MPHD after tumour surgery and CHH with pubertal arrest;)
311 compared to those with congenital causes (Kallmann syndrome, CHH with absent puberty, congenital
312 MPHD (group A: final BTV HH acquired: 50 ± 21 ml, vs. HH congenital: 34 ± 14 ; $p = 0.07$; final sperm
313 concentration acquired HH: 94 ± 81 mill/ml vs. congenital HH 43 ± 17 ; $p = 0.3$) (Table 1).

314 ***-Undescended testes***

315 Final sperm concentrations of patients with bilateral cryptorchidism were lower than for those with
316 unilateral or no undescended testis (Figure 3a). Of the whole cohort of adolescents, 15 subjects (45%
317 of group A and 32 % of group B) (Table 1) had a history of undescended testes, 4 unilateral and 11
318 bilateral at birth. All had orchidopexy before the age of six, most of them before the age of two.

319

320 ***-Initial testicular size***

321 Initial BTVs (by ultrasound) correlated with final ultrasound BTV on gonadotrophin replacement in
322 both groups (r : A:0.56/B:0.57; $p < 0.001$) (Figure 3b). BTVs also correlated with final sperm
323 concentrations (and final total counts) in group A (r : 0.51; $p = 0.025$), but not in group B.

324

325 ***-Markers of Sertoli cell maturity (inhibin B, AMH)***

326 Baseline inhibin B levels before gonadotrophin replacement correlated with final BTV in both groups
327 (r : A: 0.51/B: 0.57; $p < 0,01$) (Figure 3c). A significant correlation with final SCs (r : 0.64; $p = 0.002$) and
328 final total count (Spearman r : 0.73; $p = 0.0002$) was found only in group A.

329 There was a significant correlation of baseline AMH and final SCs and total sperm count (r : A:
330 0.42/B: 0.41; $p < 0.02$) in both groups (Figure 3d), and a significant correlation with final BTVs in
331 group A (r : 0.40; $p = 0.047$).

332

333 ***Kinetics of Sertoli cell markers during gonadotrophin replacement***

334 Group A had baseline inhibin B levels of 39 ± 35 pg/ml, rising to 76 ± 61 pg/ml on hCG alone with
335 maximum inhibin B levels on hCG/rFSH of 177 ± 118 pg/ml (normal adult range: 125-330 pg/ml)
336 (Table 1). Mean serum inhibin B levels were lower at baseline in group B with 27 ± 23 pg/ml ($p=0.02$).
337 HCG-stimulated levels (44 ± 28 pg/ml) and maximum inhibin B levels on hCG+rFSH (122 ± 73 pg/ml)
338 were not significantly different from group A.

339 Mean baseline AMH levels were not significantly different between groups A/B (respectively 31 ± 32
340 vs. 20 ± 13 ng/ml), declined to 17 ± 19 / 16 ± 15 ng/ml on hCG, and reaching minimum levels on
341 hCG/rFSH of 5.8 ± 4.3 / 3.7 ± 2.7 ng/ml (normal adult range: 1.3-14.8 ng/ml) (Table 1).

342

343 **Discussion**

344 ***Complete pubertal induction***

345 While the standard therapeutic regimen for pubertal induction in boys with HH based on testosterone
346 administration has largely neglected testicular growth and spermatogenesis, this comprehensive
347 prospective study demonstrates that induction of complete puberty including testicular maturation can
348 be achieved by gonadotrophin substitution. In addition, our observations confirm that pubertal
349 virilisation can be induced with gonadotrophins without major adverse effects and with attainment of
350 final heights in the range of mid-parental expectations in boys with HH of various origins.

351

352 ***Treatment protocols***

353 We hereby suggest a protocol for hCG/rFSH replacement in pre-pubertal boys and testosterone-
354 virilised adolescents with HH that is effective, irrespective of the underlying aetiology. While protocol
355 B (aiming at testicular maturation after completed virilisation) is comparable to regimens previously
356 described for adults^{1, 3, 4}, protocol A was established for complete pubertal induction in pre-pubertal
357 boys, allowing for developmental immaturity (including delayed bone age) and aiming to achieve
358 physiologic pubertal increments in serum testosterone levels during the first year of hormone
359 replacement via progressive hCG dose escalation.

360

361 ***Differential diagnosis of CDGP***

362 The suggested protocol A enables activation of the pubertal GnRH pulse generator in cases of
363 unrecognised constitutional delay of puberty (CDGP) by use of low initial hCG doses, exerting only
364 minimal suppressive effects on the hypothalamo-pituitary-gonadal (HPG) axis. We thereby identified
365 two patients wrongly diagnosed with HH. Nevertheless, special attention to LH levels during
366 gonadotrophin replacement seems mandatory in view of this challenging differential diagnosis.

367

368 ***Somatic outcomes (primary study end points) and duration of gonadotrophin replacement***

369 The findings of this study provide evidence that pubertal virilisation, in concert with pubertal testicular
370 growth and initiation of spermatogenesis, can be successfully induced during adolescence, with $>72\%$

371 normal (adult) final testicular sizes and >92% evidence of full spermatogenesis achieved by combined
372 treatment with hCG and rFSH. Treatment for 6 months with hCG, followed by 25 months with rFSH,
373 i.e. around 2.5 years of gonadotrophin administration seems to be required in adolescents to achieve
374 full individual potential for testicular growth and spermatogenesis.

375

376 ***Previous studies on adolescents***

377 Previous studies, including a small number of pre-pubertal HH boys have demonstrated the “proof of
378 principle” that hCG induces a rise in serum testosterone levels, resulting in virilisation^{8, 10, 26, 27}, and
379 that hCG, combined with FSH stimulates testicular growth and activates spermatogenesis in
380 adolescents^{6, 7, 8, 9, 10, 11}. Table 2 provides an overview on these studies, in comparison to our study.

381

382 ***Gonadotrophin preparations***

383 The efficacy and safety of gonadotrophin substitution in adult male HH patients for initiating testicular
384 growth and spermatogenesis, sufficient for fertility, has been reported on several occasions.^{1, 2, 3, 4}
385 HCG contains almost exclusively LH-like bioactivity¹⁸, stimulating testosterone production by Leydig
386 cells; FSH is required for spermatid maturation (spermogenesis) during the initiation, and for
387 maintenance of quantitatively normal spermatogenesis at puberty and thereafter^{19, 20}. HCG has been
388 used as a source of LH since 1952²¹ and urinary human menopausal gonadotrophin (hMG), applied to
389 substitute for FSH since 1966^{1, 2, 6, 7, 22}. Highly purified urinary FSH has been available since 1997/98
390^{2, 4}, and recombinant FSH (rFSH) since 1995^{5, 23, 24, 25}. In this study, rFSH was used, as it is the only
391 FSH preparation licenced for fertility induction in hypogonadotrophic males in Europe.

392

393 ***Arguments for conventional pubertal induction in HH***

394 An argument that has been raised in favour of the traditional replacement regimen using testosterone
395 enanthate for puberty induction in HH is the practicability of one (or two)-monthly i.m. injections and
396 the low costs of this replacement strategy. While the expenses of urinary-derived hCG-replacement are
397 comparable, rFSH is expensive. Another reason for compliance with testosterone is related to the
398 assumption that fertility is “not yet an issue” at an adolescent age and that the current strategy is
399 satisfactorily addressing the patient’s needs in terms of masculinity.

400

401 ***Impact of gonadotrophin replacement on quality of life***

402 Our results of QoL assessment pre-gonadotrophin treatment demonstrate that the feeling of “being
403 different” in terms of sexual development at a time when normal puberty occurs, has a negative impact
404 on the young HH patient’s well-being. Higher pre-treatment depression scores in boys who had
405 previously received testosterone for puberty induction (group B), compared to testosterone-naïve boys
406 (group A) indicate that replacement of testosterone does not resolve this problem. Boys with HH do
407 have pervasive and persistent concerns with body image and future fertility prospects. In support of
408 this, a recent paper identifying unmet health needs of CHH patients based on a web-based assessment

409 found that these individuals often struggle with the psychosocial sequelae of CHH²⁸. Our study
410 provides evidence for reduced anxiety and improved overall QoL parameters, when physical pubertal
411 normality is achieved, and when the potential for future fatherhood is demonstrated by activated
412 spermatogenesis.

413 Although satisfaction with testicular size was remarkably higher after gonadotrophin replacement in
414 both treatment groups, we observed lesser final satisfaction with masculinity and persistently higher
415 depression scores, even after treatment, in group B. Previous induction of incomplete puberty by
416 testosterone thus seems to neglect a “window of opportunity” to provide self-assurance and promote
417 confidence for the future. Body image and fertility concerns in the HH patient may therefore best be
418 addressed at a peer-related time. In support of this, overall compliance of study patients with taking
419 five s.c. injections per week was surprisingly good and even better in previously pre-pubertal boys
420 who were still under parental supervision.

421

422 ***Comparison of adolescent outcomes with those of adults***

423 Treatment outcomes in boys and adolescents in our trial were better than those reported for adults^{2, 4, 5,}
424 ^{19, 24} (supplementary Table 1).

425 However, higher sperm concentrations achieved by adolescents may only be a relative advantage as
426 spermatozoa of HH patients treated with gonadotrophins or GnRH have an excellent fertilising
427 potential, despite subnormal counts^{3, 8, 29, 30, 3, 32}.

428

429 ***Impact of previous testosterone replacement on somatic outcomes***

430 Our study contributes to the question whether treatment effects of gonadotrophins during adolescence
431 are affected by previous testosterone replacement. In line with a recent meta-analysis of previous adult
432 studies³³, which showed no significant association between prevalence of previous testosterone
433 replacement and sperm concentration, we did not observe differences in outcomes with hCG/FSH
434 replacement in terms of testicular size or sperm count achieved in pre-pubertal boys vs. adolescents
435 with prior full-dose testosterone replacement for up to 5.7 years. Only one out of three patients who
436 remained azoospermic had previously received testosterone. In contrast, in another study on adult
437 patients that were previously treated with androgens, a decreased likelihood of achieving sperm output
438 thresholds and conception³⁴ was observed.

439

440 ***Factors influencing therapeutic response to gonadotrophins***

441 The results of this study indicate that testicular growth potential and satisfactory sperm concentrations
442 in response to gonadotrophin substitution during adolescence depend on various factors at baseline.
443 Patients without previous bilateral cryptorchidism, with non-congenital HH causes, with higher
444 baseline testicular volumes, and with higher baseline inhibin B and AMH serum levels had more
445 favourable outcomes. These findings are in line with predictors of response to treatment that have been
446 defined in adult studies^{3, 24, 29, 31, 32, 35, 36}. All the above-mentioned parameters reflect the degree of

447 seminiferous tubular maturation that may have occurred pre-treatment, which in turn is dependent on
448 the onset and the extent of GnRH and/or gonadotrophin deficiency. However, as responses also
449 depend on adherence to treatment, individual outcomes cannot reliably be predicted. The variability in
450 observed response within certain diagnostic subgroups (Kallmann syndrome, CHH, MPHD
451 congenital) may also be due to genetic heterogeneity and oligogenicity³⁷ or epigenetic phenomena,
452 resulting in different hormone secretion patterns, varying from complete absence of pulses to disorders
453 of amplitude and/or frequency³⁸.

454

455 ***Further arguments for early gonadotrophin substitution***

456 Timely completed testicular maturation has further considerable advantages: First, it is likely to
457 significantly reduce the time necessary for re-induction of spermatogenesis by future gonadotrophin
458 cycles in adulthood^{3, 32}, thereby enabling earlier spontaneous conception of the partner. This seems
459 important in view of increasing female age at first pregnancy in modern societies. Second, poor
460 responders to gonadotrophin replacement during adolescence may respond worse with increasing age.
461 Our adolescents were therefore given the opportunity for sperm cryo-preservation, thereby
462 safeguarding a chance for future biological paternity in case of adverse future events concerning
463 fertility. Third, adolescents with persisting azoospermia may undergo mTESE before switching to
464 permanent testosterone replacement and thereby rescue sperm for potential use in future reproduction.
465 This was successfully performed in one patient in our study.

466

467 ***Future prospects***

468 Questions remain as to the optimal timing of treatment with FSH and whether an attempt should be
469 made to expand Sertoli cell numbers, either during the neonatal period (if HH is recognised by the
470 presence of micropenis and cryptorchidism)³⁹ or before initiating puberty and whether these actions
471 could improve fertility. In a recent study⁴⁰, 7 adult HH patients without cryptorchidism given FSH
472 treatment before GnRH substitution had serial testicular biopsies showing Sertoli and spermatogonial
473 cell proliferation and higher final sperm counts than in the control group. However, final SCs were far
474 below the normal range (5.8 ± 2.3 mill/ml).

475

476 ***Conclusions***

477 The availability of hCG and rFSH for replacement of gonadotrophins provides an alternative option
478 for endocrinologists to safely induce puberty in boys with hypogonadotropic hypogonadism, leading
479 to normal linear growth and virilisation, testicular enlargement and early induction of
480 spermatogenesis. This method is effective and reassuring for affected individuals and is likely to
481 reduce the expense, duration and anxiety of late fertility induction. Favourable outcomes during
482 adolescence appear not to be compromised by short-term prior testosterone substitution. Clinical
483 parameters, reflecting the onset and severity of GnRH and/or gonadotrophin deficiency may serve as
484 tools to predict response to treatment.

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Figure legends

Figure 1

a) Testicular growth over time in response to gonadotrophin replacement with hCG and rFSH in pre-pubertal (group A) and testosterone-virilised (group B) adolescents with HH.

b) Final bi-testicular volumes (BTV) over time to final BTV from start of hCG therapy in group A and B.

The dashed lines indicate the lower limit of normal BTVs (24ml); the black lines indicate mean final BTVs and mean duration from start of hCG until attainment of final BTVs.

A: mean final BTVs: 34 ± 16 ml with 74% (20/27) of patients reaching a normal BTV ≥ 24 ml; mean duration from start of hCG therapy until final BTV: 24 ± 7 months.

B: mean final BTV: 32 ± 16 ml with 70% (16/23) of patients reaching a normal BTV ≥ 24 ml; mean duration from start of hCG until final TV: 22 ± 6 months; all $p > 0,05$; n.s.

504

Figure 2

a) Increase in sperm concentration over time in response to gonadotrophin replacement with hCG and rFSH in previously pre-pubertal (group A) and previously testosterone-virilised (group B) adolescents with HH.

Baseline azoospermia was assumed in all boys of group A, as pre-treatment semen analysis was not possible due to psycho-sexual immaturity.

b) Final sperm concentration over time of rFSH treatment, until a plateau in group A and B was reached.

A: mean final sperm conc.: 40 ± 73 mill/ml, with 61% (14/23) of patients reaching a normal sperm concentration ≥ 15 mill/ml; mean duration from start of rFSH until final sperm concentration: 25 ± 7 months.

B: mean final sperm concentration: 20 ± 9 mill/ml, with 32% (6/19) of patients reaching a normal sperm conc. ≥ 15 mill/ml (A vs. B: $p = 0.007$); mean duration from start of rFSH therapy until final sperm concentration: 25 ± 9 months; (A vs. B: n.s.).

519

Figure 3

Predictors of response to hCG/rFSH treatment

522 a) Presence of undescended testes at birth: Mean sperm concentrations for patients with
523 bilateral/unilateral/no cryptorchid testes at birth were: 4 ± 6 / 38 ± 46 / 44 ± 74 mill/ml, respectively.

524 b) Correlation of initial ultrasound bi-testicular volume (BTV) with final ultrasound BTV in both
525 groups (A+B); Spearman r : 0.56/0.57; $p < 0.001$.

526 c) Correlation of baseline inhibin B serum levels with final BTV (Prader) in both groups; r : 0.51/0.57;
527 $p < 0.01$.

528 d) Correlation of baseline AMH serum levels with final sperm quality (sperm concentration and total
529 sperm count) (r : 0.42/0.41; $p < 0.02$) in both groups.

530

531 **Figure 4**

532 Box and whisker plots showing medians, interquartile ranges (boxes) and ranges (whisters) for results
533 of QoL questionnaires. These were filled in by $n = 26$ group A boys before gonadotrophin treatment
534 and again ($n = 15$) after puberty induction with gonadotrophins, while $n = 17$ testosterone-virilised
535 adolescents answered all questions prior to gonadotrophin substitution and $n = 13$ of these again
536 following gonadotrophin replacement.

537

538 **Supplementary Figure 1**

539 Self-reported satisfaction (on a score ranging from -2 to +2) with testis size and masculinity before and
540 after gonadotrophin replacement in previously pre-pubertal boys (group A) and testosterone-virilised
541 adolescents (group B) with hypogonadotropic hypogonadism.

542

543 **Supplementary Figure 2**

544 Serum testosterone levels on gonadotrophin replacement with hCG and rFSH in previously pre-
545 pubertal (group A) and previously testosterone-virilised (group B) adolescents with HH in response to
546 Leydig cell stimulation with hCG (subsequently combined with rFSH). Transient drops in levels
547 indicate omission of hCG injections by the adolescent.

548

549

550

551

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Table 1**Baseline and outcome measurements on hCG/rFSH therapy in previously pre-pubertal (A) and T-virilised (B) adolescents with HH**

Cause of HH n:A/B	baseline inhibinB (pg/ml)		baseline AMH (ng/ml)		baseline BTV (ml) Prader orchimeter/ ultrasound		cryptorchidism (% of cohort)		InhibinB max. (pg/ml)		AMH min. (ng/ml)		final BTV Prader orchio. / ultrasound (ml)		final sperm conc. (mill/ml)		final total sperm count (mill)	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Kallmann syndrome n:11/9	20±8	18±18	21±13	18±12	3.5±1.4/ 1.5±0.6	4.2±3.3/ 2.1±0.6	82	56	112±66	87±52	3.7±2.7	2.8±3.2	30±8/ 16±8	31±13/ 16±5	37±36	21±52	19±22	26±43
CHH absent puberty n:10/8	31±19	28±22	21±14	26±17	3.5±1.7/ 1.9±1.0	4.6±2.4/ 2.2±1.5	33	29	198±145	266±128	6.0±5.7	5.1±2.5	39±18/ 26±15	36±16/ 24±8	37±48	24±28	39±57	68±70
CHH pubertal arrest n:2/0	120±13	-	10±2	-	20±6/ 14.0±2.8	-	0	-	219±52	-	5.0±0	-	42±12/ 31±6	-	8.8±9	-	19±27	-
MPHD congenital n:5/3	14±1	31±33	11±5	13±6	2.5±2.0/ 0.9±0.5	6.0±5.0/ 2.6±1.1	25	33	126	133±173	3.7±2.2	2.2±2.9	23±7/ 11±6	26±23/ 24±16	19.3±20	4.2	51±16	59
MPHD after tumour n:4/6	94±102	33±29	32±14	22±11	5.5±4.4/ 4.9±6.2	6.3±4.0/ 3.4±2.8	0	0	270±156	177±35	8.7±10.9	3.9±1.5	60±28/ 36±11	24±21/ 19±9	180±222	12±12	261±303	18±12
CHARGE syndrome n:2/0	48±31	-	42±47	-	4.0±2.8/ 1.4±0.9	-	100	-	49.7	-	1.85	-	36±19 11	-	8.9±11	-	2.8±4	-

Table 1**Baseline and outcome measurements on hCG/rFSH therapy in previously pre-pubertal (A) and T-virilised (B) adolescents with HH**

all patients	39±45	27±23	31±32	20±13	4.6±4.7/ 2.7±3.8	5.0±3.4/ 2.5±1.6	45	32	177±118	122±73	5.8±4.3	3.7±2.7	35±15	32±16/ 19±8	40±73	19±38	60±160	42±55
n:34/26*																		
p-value (A/B)	p=0.02		p=0.24, n.s.		p=0.89; n.s. p=0.48; n.s.		—		p=0.14, n.s.		p=0.80, n.s.		p=0.95, n.s.		p=0.07, n.s.		p=0.43, n.s.	

*Semen was available for analysis in 23 group A and 19 group B patients

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Table 2

Outcomes of previous studies on gonadotrophin replacement in adolescents with hypogonadotropic hypogonadism and outcomes of this study

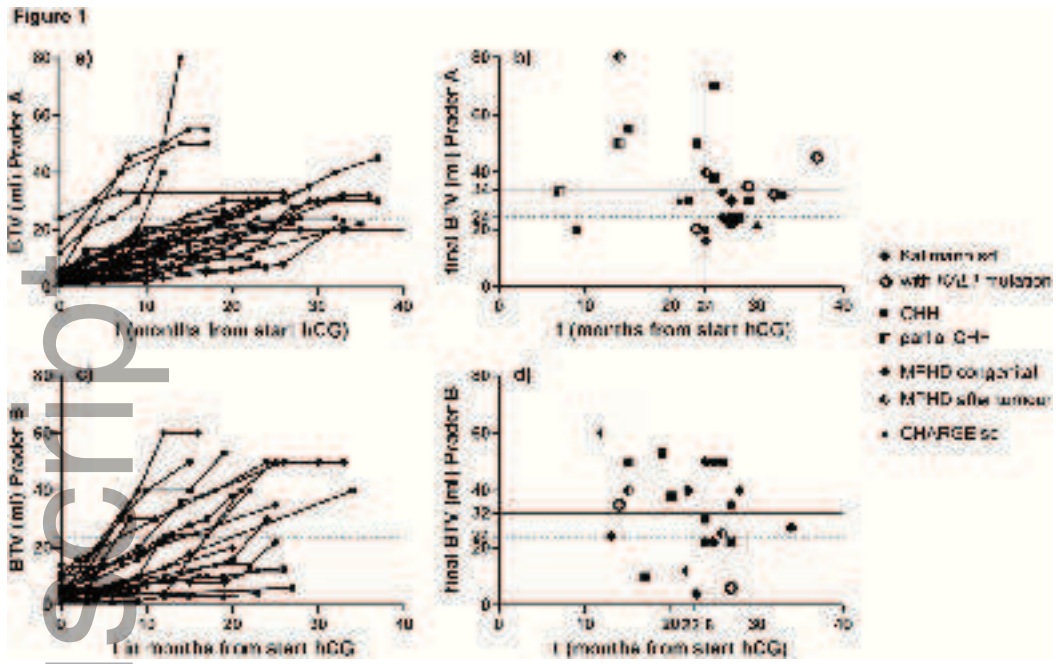
Studies	Number of adolescent HH patients	Age (years)	Gonadotrophin preparations and sequence of applications	Duration of replacement (months)	Adult T levels reached (%)	Mean±SD median(range) final single TV (ml) reached	Spermato-genesis achieved	Sperm concentration achieved (mill/ml) median (range) mean±SD	Time to sperm plateau (months)
Liu L et al. (1988) ⁶	3 (subset of cohort)	16-17	hCG/HMG	n.a.	100	9 ± 1	n.a. total cohort: 80%	<5	n.a.
Schopohl et al. (1993) ⁷	9 (subset of cohort)	18-24	hCG/MHM	20 ± 2	100	n.a. (8-30)	n.a. total cohort: 47%	n.a. (2-26)	20±2
Barrio et al. (1999) ⁸	14 IHH: 7 panhypopit: 7	13-21	hCG+rFSH	31	100	IHH: 10 ± 4 panhypopit: 15 ± 5	7/8 (87%) IHH: 4/5 panhypo: 3/3	n.a. (1.5-80)	n.a.
Raivio et al. (2007) ⁹	14	10-18	rFSH→ rFSH+hCG	rFSH: 2-34 hCG+rFSH: n.a	100	6 (2-37)	6/7 (86%)	8.5 (2.9-92)	n.a.
Sinsi et al. (2008) ¹⁰	10 (subset of cohort)	11-25	hCG→ hCG+rFSH	hCG/rFSH: 12 (-24)	100	10 (7-15)	n.a. total cohort: 81%	29 (2.6-96)	n.a.

Table 2

Outcomes of previous studies on gonadotrophin replacement in adolescents with hypogonadotropic hypogonadism and outcomes of this study

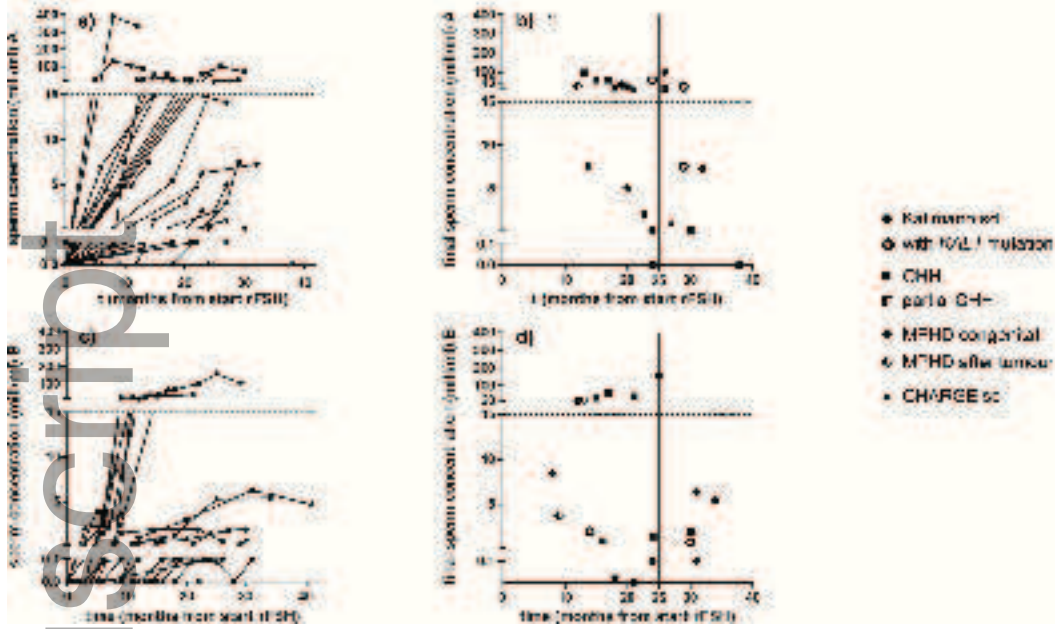
Zacharin et al. (2012) ¹¹	7 (subset of the cohort)	16-22	hCG→ hCG+rFSH	hCG/rFSH: 9	100	12 ± 7 10 (5-27)	7/7 (100%)	1.2 (0.2-15) 4.6 ± 6	n.a.
This study:	60 A:34 B:26	14-22	A: hCG→ hCG+rFSH B: Testo→ hCG→ hCG+rFSH	A: hCG: 31 ± 6 hCG/FSH: 25 ± 9 B: hCG: 30 ± 7 hCG/FSH: 25 ± 9	A: 100 B: 100	A: 17 ± 3; 15 (8-40) B: 16 ± 3 17.5 (2-30)	A: 21/23 (91%) B: 18/19 (95%)	A: 17 (0.2-337) 40 ± 73 B: 3.5 (0.1-158) 19 ± 38	A: 31 ± 6 B: 30 ± 7

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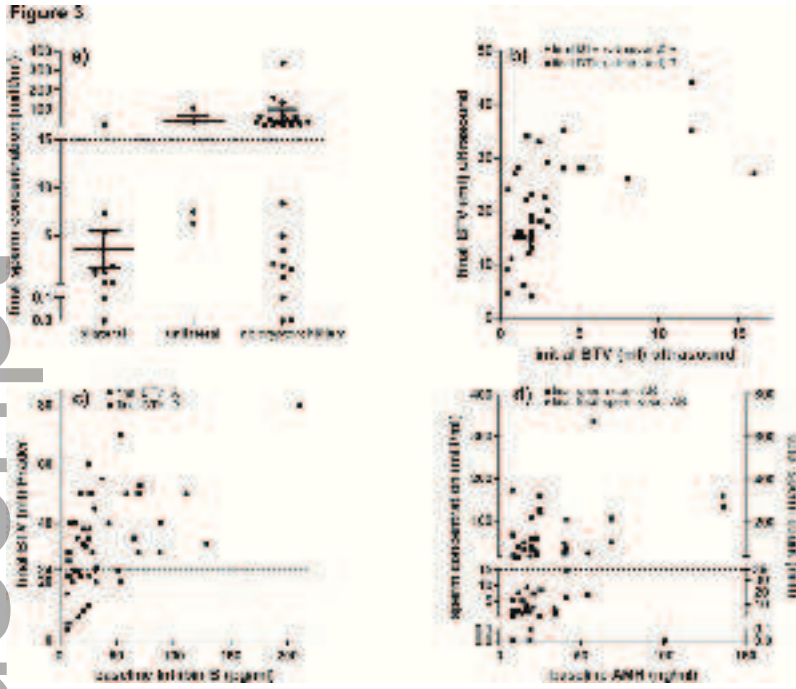
cen_13164_f1.tif

Figure 2

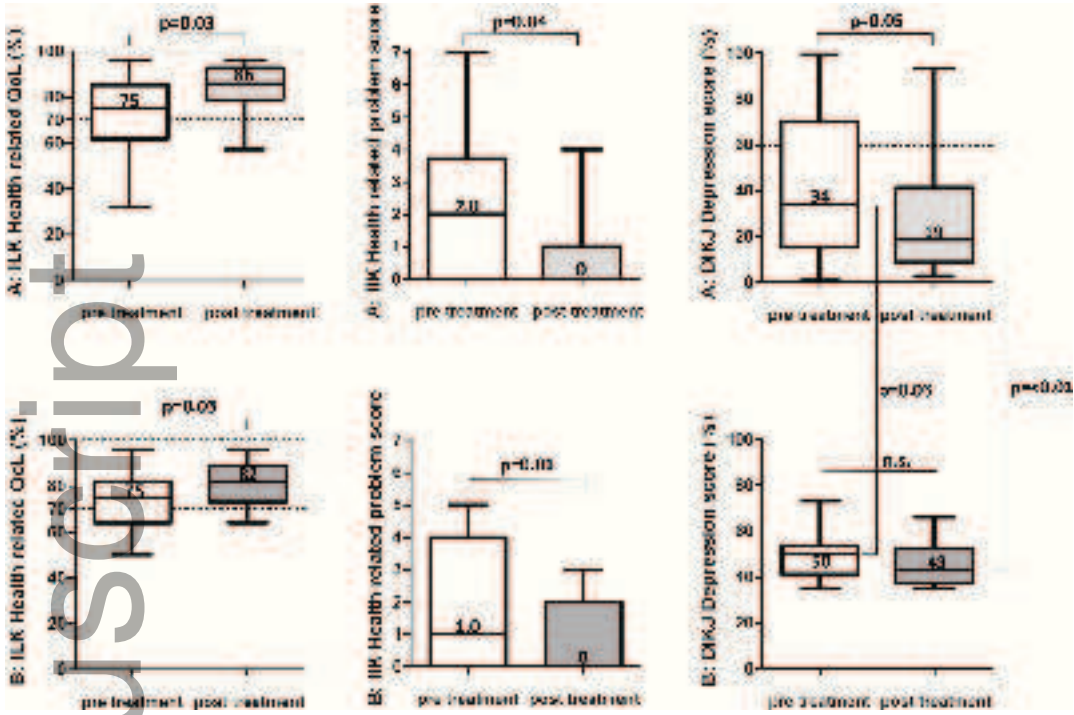


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cen_13164_f3.tif



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