1 Cushing Syndrome in a child due to Pro-opiomelanocortin (POMC) secretion from a
2 yolk sac tumour
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42 **Abbreviations:**

43 POMC - Pro-opiomelanocortin

44 ACTH – Adrenocorticotrophic hormone

45 CS – Cushing syndrome

46 EAS - Ectopic ACTH Syndrome

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Abstract

Context: Pituitary microadenomas and adrenal tumors are most common causes for endogenous Cushing syndrome (CS) in children. *Case description:* We describe a two-year old girl with Cushing syndrome due to ectopic pro-opiomelanocortin (POMC) production from an abdominal yolk sac tumor. Cortisol concentrations were elevated but adrenocorticotropic hormone (ACTH) concentrations were equivocal. The use of antibodies specifically detecting ACTH precursors revealed that plasma ACTH precursors were elevated. Additionally, an ACTH assay with a low cross-reactivity for precursors showed low concentrations of ACTH. Immunohistochemistry suggested POMC but not ACTH production by the tumor. *Conclusion:* We describe a yolk sac tumor as a novel source of ectopic POMC production leading to CS in a young girl.

What's known on this subject:

In adults, Ectopic ACTH Syndrome is most often due to intrathoracic tumors, but cases of carcinoid tumours, neuroblastoma, pheochromocytoma and carcinoma of the pancreas, thymus, thyroid and ovaries have been described.

What this study adds:

To our knowledge, this is the first report of Cushing syndrome in a child due to POMC secretion from a yolk sac tumor

Introduction

3

86   Cushing Syndrome (CS) is due to exposure to excess glucocorticoids. Clinical features

87   include obesity, impaired growth, behavioural changes, facial plethora, hirsutism,

88   muscle weakness and hypertension. Non-iatrogenic CS is rare (two to five per

89   1,000,000 per year) [1], and paediatric CS is even less common.

90   Pituitary microadenomas producing adrenocorticotropic hormone (ACTH) and adrenal

91   tumors are the most common cause of endogenous paediatric CS. Ectopic ACTH

92   Syndrome (EAS) is extremely rare and accounts for less than one percent of the cases

93   [2, 3]. In adults, EAS is most often due to intrathoracic tumors, but cases of carcinoid

94   tumours, neuroblastoma, pheochromocytoma and carcinoma of the pancreas, thymus,

95   thyroid and ovaries have been described [4]. We describe here, for the first time to our

96   knowledge, a child with CS due to ectopic ACTH precursors from an abdominal yolk

97   sac tumor.

98   In the human pituitary, POMC undergoes post-translational processing, resulting in the

99   production of pro-ACTH (further cleaved to ACTH, the N-terminal POMC fragment

100   (N-POC) and a small joining peptide) and B-lipotrophin (B-LPH) (which is cleaved to

101   produce G-lipotrophin (G-LPH) and B-endorphin (B-EP)) (Figure 1A). All peptides

102   including POMC are released into the circulation [5]. Historically, ACTH precursors were

103   identified as high molecular weight forms of ACTH in EAS tumors [6]. More recently,

104   the use of a specific two-site enzyme-linked immunosorbent assay (ELISA) using a pair

105   of monoclonal antibodies, each recognizing a specific epitope in POMC, has enabled

106   the measurement and identification of POMC [5, 7]. We used this assay to gain insight

107   into the etiology of CS in this patient.

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110   *Consent*

111 Informed and written consent were obtained from parents.

112

113 **<u>Case presentation</u>**

114 A 2.75 year old girl presented with weight gain (five kilograms in 12 months), increased

115 appetite, body odour, facial acne, pubic hair, lethargy and moodiness. She was the first

116 child of Bulgarian and Maltese parents. There was no relevant past medical history or

117 family history.

118 Her weight and height were 18.75 kg (+2.69 SDS) and 87.3 cm (-1.38 SDS) (BMI 25

119 kg/m^2). Weight gain in the last 2 months was 2.0 kg whilst height velocity was 0.7

120 cm/year. Blood pressure was 176/131 mm Hg (>> +2SD). She had a Cushingoid

121 appearance with round facies, facial acne, truncal obesity, mid-scapula fat pad and

122 hypertrichosis. The abdomen was distended without hepatosplenomegaly or palpable

123 masses. She also demonstrated proximal muscle weakness. Tanner stage was B1 P2 A1

124 M0 (Figure 2).

125 Initial investigations showed an elevated midnight serum cortisol concentration

126 (1258nmol/L) with loss of circadian variation, increased cortisol excretion in four

127 separate 24-hour urinary samples (>1380nmol/24 hours), incomplete suppression

128 (22%) of cortisol production on a low-dose dexamethasone suppression test (cortisol

129 1363 nmol/L at 0 min and 1468 and 1054 nmol/l at 24 and 48 hrs [normal < 1.8 µg/dL,

130 <50 nmol/L at 48 hrs]), and 43% suppression on a high dose dexamethasone

131 suppression test (Table 1). An ultrasound of the abdomen was normal. A brain MRI

132 showed a possible microadenoma in the left side of the pituitary.

133 The child was referred to Great Ormond Street Hospital for Children in London for

134 further investigations. A 24-hour serum cortisol profile showed no circadian rhythm

135 with increased cortisol (35.5ug/dL, 985nmol/L) and ACTH concentration (51.8ng/L,

11.5pmol/L (normal <5ng/L)) at midnight, and a morning ACTH of 39.9ng/L (8.8pmol/L, normal 10-50ng/L) (data not shown). A corticotropin releasing hormone test (CRH test) (100mcg CRH) showed a 12% increase in ACTH concentration and no clear increase in cortisol concentration from baseline (Table 2). The baseline production of other pituitary hormones was normal. Dehydroepiandrosterone (DHEAS) and androstenedione were elevated. Potassium was low normal (3.5mmol/L). The child had an episode of back pain and refused to walk. Orthopaedic investigations including spinal radiographs were normal, and she improved spontaneously.

The CRH test was inconsistent with pituitary-dependent Cushing disease and EAS was considered. A thoracic CT-scan of the chest revealed marked nodular infiltration of the peritoneal surface of the diaphragm. A repeat abdominal ultrasound examination showed a solid pelvic mass (3.5x4.3x3cm) and a solid heterogeneous infiltrating mass diffusely surrounding the liver and spleen (mimicking prominent adipose tissue) suggesting peritoneal infiltration by the tumor. Lymphadenopathy was present at the level of the porta hepatis. Abdominal MRI (Figure 2) confirmed findings in keeping with malignant infiltrating peritoneal disease. Both adrenals appeared bulky without focal lesions. Alpha-feto protein (AFP) concentration was grossly elevated (>300,000 kU/L) whereas B-HCG (human chorionic gonadotropin) was undetectable. A ^{99}Technetium scan showed increased uptake in multiple ribs and vertebral bodies, femur and humerus compatible with metastatic bone disease. Bone marrow aspiration was normal.

Tumour needle biopsy demonstrated a primitive malignant epithelial tumour with no specific morphological features, expressing AE1/3 (cytokeratin), but CD117, Oct3/4, CD56, desmin, AFP, WT1 and S100 staining were negative. The child received Metyrapone and Ketoconazole, which successfully suppressed cortisol production.

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161 Further treatment consisted of five courses of Cisplatin, Doxorubicin, and Etoposide,

162 which led to reduction of AFP concentrations to 1264kU/L and of cortisol production,

163 so that Metyrapone and Ketoconazole could be stopped. The patient had multiple

164 episodes of sepsis and neutropenia, and on one occasion signs of possible thrombosis,

165 but this improved spontaneously. Adrenal function was suppressed and she required

166 hydrocortisone replacement therapy. However, AFP concentrations increased again.

167 Surgical resection was attempted, but multiple tumor plaques over the peritoneum and

168 intra-abdominal organs prevented complete resection. AFP concentration fell from

169 17654kU/L prior to surgery to 6590kU/L post-surgery. Histological examination of

170 resected tissue demonstrated sheets of malignant epithelial tumor expressing MNF116,

171 CEA, and AFP but not other markers such as desmin, vimentin, inhibin, WT1,

172 calretinin, CD56, Oct3/4 and CD17; the overall features were strongly suggestive of a

173 malignant yolk sac tumor. The tumor was graded as Grade IV (distant metastases).

174

175 **Methods**

176 **Identification of ectopic ACTH precursor production**

177 *Plasma ACTH and ACTH precursors*

178 Plasma ACTH was measured initially with a solid phase two-site chemiluminescent

179 immunometric assay (Immulite 2000, Siemens) during diagnostic investigations. This

180 assay has a sensitivity of 5ng/L (1pmol/L) and an inter-assay variability of 6-10%.

181 Cross-reactivity with ACTH precursors in this assay has recently been assessed to be

182 2.2 % (MONAGHAN 2016) . After the first and second cycle of chemotherapy, ACTH

183 and ACTH precursors (POMC and pro-ACTH) were measured by ELISA (in house)

184 using the monoclonal antibodies N1C11 and A1A12. Binding of both antibodies to the

185 ACTH precursors is required to generate a signal in the assay; therefore, ACTH or any

186   of the other peptides derived from POMC and proACTH are not detected [8]. The

187   sensitivity of the precursor ELISA is 8pmol/L and normal adult range of precursors is

188   7-32pmol/L [9]. Circulating concentrations of ACTH precursors above 100pmol/L are

189   indicative of an ectopic tumour [5, 10, 11]. Measurement of ACTH utilises MAb A1A12

190   which binds to ACTH (10-18) and MAb A2A3 which binds the cleavage site of ACTH

191   and therefore reduces the cross-reactivity with ACTH-precursors (Figure 1A) [7]. This

192   ACTH ELISA has a sensitivity of 1 pmol/L, variability <10% and POMC cross-reacts

193   <3% (unpublished data).

194   *Immunohistochemistry for ACTH and precursors*

195   Paraffin sections of tumour biopsy were evaluated following antigen retrieval with

196   mouse monoclonal antibodies A1A12, N1C11, E6B2 and A2A3, which recognise

197   various epitopes of POMC and ACTH as described previously [10] (Figure 1A). Staining

198   was visualized using a Mouse Dako Envision+ System-HRP (DAB) and Gill's

199   haematoxylin counterstain. Controls were rat pituitary sections (ACTH and POMC

200   positive) and a DMS79 small cell lung cancer xenograft tumour [10], known to be POMC

201   positive and ACTH negative.

202

203   **Results**

204   *Plasma ACTH and ACTH precursors*

205   In the first blood sample, taken before the second course of chemotherapy, ACTH

206   precursors were detected at a concentration exceeding the normal adult range (7-

207   32pmol/L) [9] whereas ACTH concentrations (measured with the matching ACTH

208   ELISA) were within the adult normal range. In the second sample, taken just before the

209   3rd cycle of chemotherapy, the ACTH precursor concentration had decreased

210   considerably (Table 3). A small amount of "ACTH" was detectable in both samples. It

211 is very likely that this is due to the low cross-reactivity of ACTH precursors in the

212 ACTH assay or endogenous ACTH secreted from the pituitary.

213 *Immunohistochemistry for ACTH and ACTH precursors in tumor sections*

214 The tumor showed strong cytoplasmic staining with antibodies A1A12 (recognizes

215 ACTH and ACTH precursors), N1C11 (recognizes N-POC and ACTH precursors) and

216 E6B2 (recognizes beta-endorphin and POMC) but not with the more specific ACTH

217 antibody (A2A3) (Figure 1B). This suggests the presence of POMC, but not mature

218 ACTH, in tumor cells. Immunohistochemistry using A2A3 in a control POMC-

219 producing xenograft tumor did not show any staining (data not shown), confirming

220 specificity of A2A3 for mature ACTH.

221 *Further treatment*

222 After establishment of the diagnosis of yolk sac tumour, treatment was changed to

223 Paclitaxel, Ifosfamide and Cisplatin (TIP, 3 courses), after which AFP concentration

224 fell to 2340kU/L and tumor size decreased to 2.7x3.2 cm with only discrete areas of

225 disease intraperitoneally. A further TIP course and high-dose chemotherapy (Paclitaxel,

226 Carboplatin, Etoposide, Cyclophosphamide) with autologous peripheral blood stem cell

227 rescue was given. Despite this, AFP increased again (26444kU/L). Hyperthermic

228 Intraperitoneal Chemotherapy (HIPEC) was administered without long-term success.

229 She further received Gemcitabine, Docetaxel and Metronimic chemotherapy with

230 Cyclophosphamide and Etoposide [12]. Whole abdominal radiotherapy in a dose effective

231 for treatment of germ cell tumours (54Gy) was not possible to deliver. She then

232 continued to receive palliative chemotherapy and unfortunately passed away 1.5 years

233 later.

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240 **<u>Discussion</u>**

241 We describe, for the first time to our knowledge, POMC production from a malignant

242 yolk sac tumor as the cause of CS due to 'ectopic ACTH precursor production' in a

243 child. Yolk sac tumors (endodermal sinus tumors) are a malignant subtype of germ cell

244 tumor [13, 14], characterized typically by AFP and Gli-3 immunoreactivity and may occur

245 in both gonadal and extra-gonadal tissues. Reports of EAS in adults due to ACTH

246 production in teratoma [15, 16], ovarian epithelial [17, 18] and ovarian endometrial carcinoma

247 [19] exist but EAS has not previously been described in a yolk sac tumor. Two large case

248 series of 90 adults at NIH and 40 adults in London with EAS have recently been

249 published, but yolk sac tumour was not identified as a cause for EAS [20, 21].

250 Another recent case series described ten children with EAS identified during the last 20

251 years in France [22]. Seven patients had thoracic neuro-endocrine tumors; one had a liver

252 nested stromal epithelial tumor, one a carcinoma of the thymus and one Ewing's

253 sarcoma. Of note, positive ACTH staining of the tumour was one of the inclusion

254 criteria. Therefore, POMC/pro-ACTH producing tumours may not have been included,

255 depending on the antibodies used for ACTH detection.

256 To identify the etiology of the CS in our patient, four different antibodies were used in

257 plasma ELISAs and tumor immunohistochemistry. The ACTH precursor ELISA,

258 which measures both POMC and pro-ACTH, gave the first indication that plasma

259 ACTH precursor concentrations were increased. IHC staining of the tumor with

260 A1A12, N1C11 and, also, E6B2, which binds to POMC but not pro-ACTH, showed

261 positive staining. This is strong evidence that the tumor produced POMC but did not

262 cleave it to mature ACTH. Additionally, the tumor did not stain with A2A3, which is

263 specific for mature ACTH. In addition, the POMC concentration decreased after

264 chemotherapy, in line with reduction in tumor size and improvement of features of CS.

265 These data suggest that either POMC is binding to the ACTH receptor (melanocortin-

266 2 receptor, MC2-R), in the adrenal gland to stimulate cortisol secretion or that POMC

267 is cleaved in the adrenal gland to allow ACTH to bind and stimulate cortisol secretion.

268 Indeed, other patients with ectopic "ACTH" syndrome due to ACTH precursor

269 production have clinical symptoms [23, 24], also suggesting that ACTH-precursors bind to

270 the adrenal MC2-R or are locally cleaved. While it has not been possible to investigate

271 the bioactivity of POMC because of the high concentrations of purified POMC that is

272 required, we have shown that POMC can bind to the MC1-R [25].

273 Differentiation between an ACTH-producing pituitary adenoma (Cushing Disease, CD)

274 and EAS can be difficult. No single biochemical test can differentiate between the two

275 and responses to dynamic function tests overlap. Bilateral inferior petrosal sinus

276 sampling (BIPPS) [22] remains the golden standard to differentiate pituitary Cushing and

277 EAS (Lacroix, Lancet 2015), but it is difficult to perform, particularly in children. This

278 case illustrates the potential usefulness of measurement of ACTH-precursors in order

279 to differentiate between CD and EAS. Peripheral concentrations of ACTH-precursors

280 are low in CD and high in EAS and a clear relation exists between BIPSS results and

281 baseline ACTH precursor concentration [7]. Stewart et al, 1994 ACTH precursor

282 assessment (by immunoradiometric assay) showed a 100% sensitivity and specificity

283 in a group of EAS patients and CD patients. Levels of ACTH precursors were between

284 139 and 18,000pmol/L in EAS patients, between 8 and 73pmol/L in the CD patients

285 and below 40pmol/L in control subjects. Measuring ACTH precursors can also

286 distinguish the majority of occult ectopic 'ACTH' producing tumours that are not

287 detected by MRI. Page-Wilson et al, 2014 (reference 9) showed that in their patient

288 cohort 7 of 11 such patients could be distinguished by ACTH precursor measurement

289 alone. This gives a sensitivity of 64% and 100% specificity.

290

291 Yolk sac tumors are chemosensitive but when complete resection is not feasible, and

292 there is cisplatin resistance, relapse is frequent and the outcome is poor, as seen in this

293 case [26]. Medical treatment of EAS with Metyrapone and Ketoconazole is effective in

294 decreasing cortisol production, but is of limited use due to side effects.

295

296 Complications of EAS are increased susceptibility for infections and thrombosis.

297 Therefore, if possible, chemotherapy is started only when cortisol production is

298 controlled pharmacologically. Antibiotic prophylaxis, PCP prophylaxis and anti-

299 coagulation could be considered, but currently no guidelines or evidence base exists for

300 paediatric EAS.

301

302 To conclude, we used antibodies to different epitopes on ACTH-precursors in ELISAs

303 and immunohistochemistry to demonstrate, for the first time, POMC production in a

304 disseminated malignant yolk sac tumor as the underlying cause of CS in a two-year-old

305 child. Hence, a diagnosis of aggressive Cushing syndrome in the face of normal ACTH

306 concentrations should prompt a search for ACTH precursors in EAS.

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385 **<u>Authors' contributions</u>**

386 EG-writing the manuscript, literature search, data collection and interpretation; SM-

387 writing the manuscript, figures and carrying out immunohistochemistry; PS-writing the

388 manuscript, data collection and interpretation; JT-writing the manuscript; CP-writing

389 the manuscript; NS-writing the manuscript and providing histopathology report; OS-

390 writing the manuscript and clinical management of the patient; AW-writing the

391 manuscript, data collection, figures and carrying out immunohistochemistry; MTD-

392 writing the manuscript, literature search, data collection and interpretation, figures.

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409 **Tables**

410 **Table 1 - Low dose dexamethasone and high dose dexamethasone suppression tests**

411 **Table 2 - Corticotropin releasing hormone (CRH) test (100 mcg CRH)**

412 **Table 3 - Plasma concentration of ACTH precursors and ACTH after first and**

413 **second cycle of chemotherapy**

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415 **Legends to figures**

416 **Figure 1 - POMC but not ACTH is produced by the tumor.**

417 **1A:** Antibodies against epitopes in ACTH and ACTH precursors used in ELISAs and

418 immunohistochemistry.

419   1**B:** Immunohistochemistry of tumor sections using mouse antibodies to ACTH-related

420   peptides and horseradish peroxidase conjugated anti-mouse IgG. Staining with A1A12,

421   N1C11 and E6B2 recognizing epitopes on ACTH precursors is positive (brown stain)

422   but staining with A2A3, specific for ACTH, is negative. Primary antibody is omitted in

423   the negative control.

424   **Figure 2 - Clinical features of the patient described. A-C** Clinical photographs

425   showing Cushingoid features at time of diagnosis. **D** MRI of the abdomen showing

426   tumor and tumor invasion in peritoneum.

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433   **References**

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