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List of abbreviations:

BMI	Body mass index
CT	Computerised tomography
GCS	Glasgow Coma Scale
GHD	Growth hormone deficiency
HPA	Hypothalamic-pituitary-adrenal
ISS	Injury Severity Score
ITT	Insulin tolerance test
KOSCHI	King's Outcome Scale for Childhood Head Injury
SDS	Standard deviation score
TBI	Traumatic brain injury

Abstract

Objectives: Post-traumatic hypopituitarism is well described among adult traumatic brain injury survivors. We aimed to determine the prevalence and clinical significance of pituitary dysfunction after head injury in childhood.

Design: Retrospective exploratory study.

Patients: 33 survivors of accidental head injury (27 males). Mean (range) age at study was 13.4y (5.4-21.7y) and median (range) interval since injury, 4.3y (1.4-7.8y). Functional outcome at study: 15 good recovery, 16 moderate disability, two severe disability.

Measurements: Early morning urine osmolality and basal hormone evaluation were followed by the GnRH and insulin tolerance (n=25) or glucagon tests (if previous seizures, n=8). Subjects were not primed. Head injury details were extracted from patient records.

Results: No subject had short stature (mean height SD score +0.50, range -1.57 to +3.00). Sub-optimal GH responses (<5 µg/L) occurred in six peri-pubertal males (one with slow growth on follow-up) and one post-pubertal male (peak GH 3.2 µg/L). Median peak cortisol responses to insulin tolerance or glucagon tests were 538 and 562 nmol/L. 9/25 and 2/8 subjects had sub-optimal responses respectively, two with high basal cortisol levels. None required routine glucocorticoid replacement. In three, steroid cover was recommended for moderate/severe illness or injury. One male was prolactin deficient. Other basal endocrine results and GnRH-stimulated LH and FSH were appropriate for age, sex and pubertal stage. Abnormal endocrine findings were unrelated to the severity or other characteristics of TBI, or functional outcome.

Conclusions: No clinically significant endocrinopathy was identified amongst survivors of accidental childhood TBI, although minor pituitary hormone abnormalities were observed.

Introduction

The hypothalamus and pituitary are vulnerable to vascular and mechanical injury as a consequence of trauma.[1] Cross-sectional and prospective longitudinal studies have reported long-term hypopituitarism in between 11% and 69% of adult traumatic brain injury (TBI) survivors.[2] Whilst many early endocrine abnormalities resolve within months of injury, others may emerge later.[2] Growth hormone deficiency (GHD) is commonest, reported in between 10% and 38% of subjects.[2] In a recent meta-analysis,[3] the pooled prevalence of anterior pituitary dysfunction ≥ five months after adult TBI was 27%. Early data suggest that post-traumatic hypopituitarism may impair physical rehabilitation and functional outcome after TBI.[4] Consensus guidelines recommend screening adult TBI survivors for pituitary dysfunction,[5] including GH stimulation testing ≥12 months after injury.[6]

Post-traumatic hypopituitarism has been reported in preliminary paediatric TBI studies.[7,8,9] However, there is limited understanding of the likelihood, causative mechanisms and significance of pituitary dysfunction in this group. We report a retrospective exploratory study investigating the prevalence and clinical significance of post-traumatic hypopituitarism amongst a cohort of childhood TBI survivors. We hypothesised that clinically significant pituitary dysfunction would be as prevalent among children as in adults. We also aimed to identify any head injury characteristics associated with post-traumatic hypopituitarism.

Subjects & Methods

Subjects

The study population comprised subjects with previous accidental TBI, aged <16 years at injury and admitted to Edinburgh hospitals between 1st January 2001 and 31st August 2007. Inclusion criteria were 1) significant head injury, defined as (a) moderate or severe TBI, or (b) minor TBI with injuries incurring an Injury Severity Score (ISS) ≥16 (see below); and 2) a minimum interval of 12 months between head injury and study entry. Exclusion criteria

included: death during or after admission, residence outside Scotland or north-east England, missing case notes, and any history of neurodevelopmental delay, cerebral palsy, learning disability or pituitary dysfunction preceding TBI. Individuals with significant physical and/or learning disability as a consequence of TBI were not excluded provided that informed consent could be obtained from the subject or a parent/legal guardian acting in their best interest.

Eligible subjects were identified by the Information and Statistics Division of the Scottish Health Service using International Classification of Diseases (ICD-10) diagnostic codes for head injury. Eligibility was verified by examining patients' computerised admission details. All subjects (or their parents/guardians if aged <16) provided informed consent prior to study entry. The Scotland Multi Research Ethics Committee A approved the study.

Age at injury was obtained from subjects' medical records. We used post-resuscitation Glasgow Coma Scale (GCS) scores to grade TBI severity. Minor, moderate and severe TBI were defined respectively as head trauma with post-resuscitation GCS scores of 13–15, 9–12 and <9.[10] The ISS is an anatomical scoring system providing a score from 0–75 for patients with multiple injuries.[11] An ISS score of ≥16 was chosen to enable inclusion of 'minor' TBI subjects with extradural or subdural haemorrhages who were intubated and ventilated for neurosurgery before their GCS score fell below 13.

We reviewed subjects' cranial computerised tomography (CT) scans to identify injuries that might be associated with pituitary dysfunction. The Marshal CT score[12] was used to grade diffuse brain injury. We documented any evidence of intra-cerebral oedema (± midline shift) on imaging and any history of post-traumatic seizures.

Clinical assessment at study

A medical history was taken from each subject. We evaluated functional outcome after TBI using the King's Outcome Scale for Childhood Head Injury (KOSCHI).[13] Height and weight

were measured and used to determine body mass index (BMI). Parent-reported parental heights were also recorded. We determined standard deviation scores (SDS) for anthropometric measurements using the Pfizer International Growth Database (KIGS) Auxology Calculator (Pfizer Endocrine Care™). This uses Tanner/Whitehouse reference data for height and height velocity[14] and the British 1990 standards produced by the LMS method for weight and BMI.[15,16] We performed Tanner pubertal staging[14] where clinically indicated, with subjects' consent. Subjects were grouped into three categories: pre-puberty (stage B1 in girls, or G1 in boys with testicular volumes <4 mL), early/mid-puberty (B2–3 in girls or G2–3 in boys) and late/post-puberty (B4–5 in girls or G4–5 in boys).

Endocrine assessment

Each subject fasted from 24:00h the night before and provided an early morning urine sample for osmolality at assessment. A basal (08:00-10:00h) blood sample was obtained for plasma cortisol, IGF1, TSH, free T4, prolactin, urea, electrolytes, creatinine and osmolality.

We measured GH and cortisol responses to stress using the insulin tolerance test (ITT) as first-line or the glucagon test in subjects with previous seizures. Peri-pubertal subjects were not primed with sex steroids before testing. In both tests, baseline samples were obtained for serum GH, cortisol and laboratory glucose at -30 minutes and time 0. For the ITT, short acting insulin was administered intravenously at time 0 at a dose determined by the baseline point-of-care glucose meter reading: 4.0–4.5 mmol/L, 0.1 u/kg; >4.5 mmol/L, 0.15 u/kg. Further blood samples for GH, cortisol and meter and laboratory glucose were taken at 20, 30, 60 and 90 minutes post-injection. Subjects received a glucose-containing drink after achieving adequate hypoglycaemia (blood glucose ≤2.2 mmol/L). For the glucagon test, 20 µg/kg (maximum 1 mg) of glucagon was administered intramuscularly at time 0. Blood samples for GH, cortisol and meter and laboratory glucose were taken at 20, 60, 90, 120, 150 and 180 minutes.

LH and FSH responses to stimulation were evaluated using the low-dose GnRH test.[17] We collected blood samples for LH, FSH and oestradiol or testosterone at time 0. Further samples for LH and FSH were obtained at 20 and 60 minutes post-intravenous injection of 10 µg of GnRH.

Analytical methods

Urea, creatinine, electrolytes, osmolality and glucose were measured by standard laboratory methods. All hormones were measured by automated chemiluminescent immunoassays. Free T4, TSH and oestradiol were measured using the Architect analyzer (Abbott Diagnostics, Maidenhead, UK). Respective coefficients of variation were <6%, <3% and 14% (at 70 pmol/L oestradiol). LH, FSH, prolactin, cortisol and testosterone were measured using the ADVIA Centaur analyzer (Siemens Healthcare Diagnostics UK). Coefficients of variation were <3%, <3%, <5%, <6% and <9% respectively. GH was measured using an immunoassay calibrated against IS 98/574 on the Immulite 2000 analyzer (Siemens Healthcare Diagnostics UK) with a coefficient of variation of <5%. IGF1 levels were measured by a two-site immunoenzymometric assay (OCTEIA IGF1) supplied by Immunodiagnostic Systems Ltd, Tyne and Wear, UK, with a coefficient of variation of <11%.

Interpretation of endocrine test results

We interpreted IGF1 concentrations in relation to age- and sex-related reference ranges determined for the Nichols Advantage assay (Nichols Institute Diagnostics, San Clemente, California, USA),[18] which gave good agreement with the OCTEIA assay used in this study (M. Wallace, personal communication). Other basal results were interpreted using locally established paediatric reference ranges.

We defined an adequate peak GH response to insulin-induced hypoglycaemia or glucagon as >5 µg/L. The ITT cut-off was established locally using data from Scottish children and the Immulite 2000 GH assay. The same cut-off was used for the glucagon test. We used locally

determined age-dependent cut-offs to evaluate peak cortisol response to the ITT: ≥470 nmol/L in subjects ≥10 years; ≥550 nmol/L in subjects <10 years.[19] A cut-off of 450 nmol/L was used to define an adequate peak cortisol response to glucagon.[20] We evaluated peak LH and FSH responses to the low-dose GnRH test using locally determined reference data.[17]

Statistical analysis

Continuous data were expressed as mean (range) if Gaussian or median (range) if non-Gaussian. Comparisons between groups were performed using the two-sample t-test or the Mann-Whitney U test as appropriate. The Chi-squared or Fisher's exact tests were used to compare categorical data. Statistical comparisons were performed using Minitab version 14.

Results

The study population comprised 154 subjects aged <16 years at injury. One hundred and thirty-three met eligibility criteria. One hundred and two subjects were invited to enter the study (77%); reasons for exclusion are listed in Figure 1. Thirteen subjects with GCS scores of 13–15 were excluded despite small subdural or extradural haemorrhages with an ISS of 16 because they remained clinically well and did not require mechanical ventilation or neurosurgery. We concluded that the benign clinical course did not justify invasive testing. Nine others with extradural haemorrhages were excluded because head injury severity and clinical course were unclear from computer records and case records were unavailable. Thirty-four subjects consented to enter the study but tests were cancelled in one case because secure venous access could not be established. Hence, results are available for 33 subjects.

We compared characteristics of recruited subjects with those of the wider cohort to determine how representative our sample was. Males comprised 76% of recruited subjects and 76% of all eligible subjects. Mean age at injury was similar in invited (9.5y), uninvited (9.7y), recruited (9.2y) and non-recruited subjects (9.6y, all p ≥0.6). Mean age at study was

comparable between recruited and non-recruited subjects: 13.4 (5.4–21.7) vs. 13.5 (2.0–21.0) years (p=0.9). More subjects with minor TBI (5/33 vs. 5/69) and fewer with severe TBI (12/33 vs. 31/69) were recruited than not, but differences were not statistically significant (p=0.2).

Sample characteristics

Characteristics of recruited subjects are summarised in Table 1. No subject had clinical evidence of short stature or abnormal pubertal development. Mean height, weight and BMI SDS were comparable and within population norms. Target height and parental-adjusted height values were within ±2.0 SD for all but one subject (data not shown). One child was on long-term (supra-physiological) corticosteroid therapy for an unrelated medical condition (omitted on the day of testing); two others were prescribed low-dose inhaled steroids for asthma.

Endocrine evaluation

Pituitary hormone abnormalities were identified in 13 of 33 subjects (39%), involving two hormone axes in four subjects (12%). Further details are provided below and in Table 2.

Basal hormones: There was no evidence of diabetes insipidus from paired early morning urine and plasma osmolalities (n=32). No subject had central hypothyroidism. TSH was mildly raised in one child (5.2 mU/L, reference range 0.5–4.2 mU/L), raising the possibility of an unrelated thyroid disorder. Repeat testing was advised in six months. One male subject was prolactin deficient (<50 mU/L, reference range 60–500 mU/L). Two subjects had mildly reduced IGF1 concentrations for age and pubertal stage: 146 µg/L at 12.6 years and 179 µg/L at 14.8 years (reference ranges 150–600 µg/L and 200–650 µg/L for ages 11–14 years and 14-17 years respectively). The first had a normal GH response to the ITT; the other's response was suboptimal (peak GH 2.8 µg/L; subject 9, Table 2).

Hypothalamo-pituitary-gonadal axis: Basal oestradiol or testosterone and peak LH and FSH responses to GnRH were appropriate for age, sex and pubertal stage in all subjects.

HPA axis: Median basal cortisol was 298 (110–722) nmol/L (reference range 06:00h–10:00h: 150–600 nmol/L). Levels were slightly low in two subjects (110 and 146 nmol/L). Twenty-five subjects underwent an ITT, and eight, the glucagon test (all with previous seizures). Plasma glucose fell to ≤2.2 mmol/L in all subjects undergoing an ITT. Median peak cortisol was 538 (367–717) nmol/L during the ITT and 562 (289–729) nmol/L during the glucagon test. Peak cortisol responses to the ITT were suboptimal in nine subjects (Figure 2A), including the two with low basal cortisol levels (see above). Two were on low-dose inhaled steroids; the subject on high-dose steroid therapy had a normal cortisol response to stimulation. Five of nine had only borderline low responses (within 50 nmol/L below the cut-off) requiring no treatment. In three others, peak cortisol response was between 50 and 100 nmol/L below the cut-off and steroid cover was recommended during moderate or severe illness or injury. The ninth had a high basal cortisol (624 nmol/L). Two of eight subjects had flat cortisol responses to glucagon. In one case, basal cortisol concentration was high (722 nmol/L). The other subject (subject 8, Table 2) declined further evaluation of the HPA axis; no treatment was recommended.

GH axis: Median peak GH response to stimulation was 7.9 (2.5–25.4) µg/L. Seven males had sub-optimal peak GH responses (<5 µg/L) to the ITT (n=6) or glucagon (n=1) (Figure 2B). Six were in late pre-puberty or early to mid-puberty. All had height SDS within the range -1.52 to 1.38. Four of these had normal height velocities in relation to pubertal stage at follow-up (>-0.8 SD in all cases); in the fifth (subject 9, Table 2), growth velocity was inappropriately slow and GH was prescribed. The sixth failed to attend two follow-up appointments. The seventh subject (subject 3, Table 2) was post-pubertal, with a borderline low GH response to the ITT (3.2 µg/L, height SDS -0.93). Consensus guidelines variously use cut-offs of <3 µg/L[21] or <5 µg/L to the ITT[22] to define GHD in adolescence.

Anthropometry in relation to GH status:

We excluded results for the subject on supra-physiological corticosteroid therapy. Comparisons were made between subjects with normal (n=25) and suboptimal (n=7) GH responses to stimulation testing. There were no significant differences in SDS between normal and abnormal groups with respect to median height (0.56 vs. 0.75, p=0.7), weight (0.57 vs. 1.20, p=0.5) or BMI (0.52 vs. 1.30, p=0.1).

Hormone outcome in relation to head injury characteristics:

Subjects with normal (n=20) and abnormal (n=13) endocrine results were similar with respect to: mean age at injury (9.2y vs. 9.3y, p=0.9), age at study (13.5y vs. 13.2y, p=0.9), interval since TBI (4.3y vs. 3.9y, p=0.5), and TBI severity (p=0.3). Nine of 12 (75%) survivors of severe TBI had normal pituitary function.

No subject had radiographic evidence of hypothalamo-pituitary injury. Eight subjects had no visible intracranial pathology on CT scan, of whom five had endocrine abnormalities. The only subject with a diencephalic injury (a thalamic haemorrhage) had a suboptimal peak GH response to the ITT (3.2 µg/L; subject 3, Table 2). Both subjects with diffuse axonal injury had normal endocrine function. Similar proportions of subjects in both endocrine outcome groups had sustained a basal skull fracture, frontal lobe injury, extra-axial haemorrhage or intra-parenchymal clot or contusion, developed intra-cerebral oedema ± midline shift on CT scan, or suffered post-traumatic seizures (all p ≥0.3). There was no evidence of an association between a Marshall CT score >1 and pituitary dysfunction (p=0.2).

In the two subjects who were severely disabled at study, one had normal and one, abnormal pituitary function (subject 3, Table 2). In the moderately disabled group, ten had normal pituitary function and six had pituitary hormone abnormalities.

Discussion

This cross-sectional study examined the prevalence of pituitary dysfunction in a cohort of British childhood TBI survivors. We observed pituitary hormone abnormalities in 13/33 subjects (39%), affecting two axes in four cases. However, none were unequivocally clinically significant.

Eleven subjects had suboptimal cortisol responses to stimulation, discounted in two because pre-test cortisol levels were high, indicating normal HPA function. Although a further two were on inhaled steroid therapy, the low doses made adrenal suppression unlikely. Rather, our results raise the question of stimulation test reproducibility. Peak cortisol responses to the ITT are reproducible in healthy adults but there may be within-subject variability in patients with subtle HPA dysfunction.[23] Few with severe ACTH deficiency are misclassified.[23] Two of our subjects with suboptimal cortisol responses had undergone the glucagon test, which is known to elicit a poor cortisol response in approximately 10% of healthy individuals.[20] No subject had symptoms or a low enough peak cortisol response to suggest severe ACTH deficiency. Partial HPA insufficiency is poorly defined and approaches to treatment vary widely.[24] Under normal (unstressed) physiological conditions, such patients have a cortisol day curve similar to controls[25,26] and conventional hydrocortisone replacement regimens may be excessive. We recommended hydrocortisone cover during illness for three subjects.

Most (6/7) of our low GH responses to the ITT could be due to peri-pubertal blunting. These subjects showed no evidence of growth impairment at assessment and remain under review. We initiated GH therapy in one, in whom height velocity at follow-up was inappropriately slow for pubertal stage despite findings consistent with resolving constitutional delay of growth and puberty. We will reassess the GH axis off treatment at adult height. The decision not to prime peri-pubertal subjects with exogenous sex steroids prior to GH stimulation testing contrasted with our normal clinical practice. Asking subjects to attend the research

centre for pubertal staging several days before their endocrine assessment would have been difficult for families coming from afar. Priming can temporarily reverse physiological blunting of GH secretion in late pre-, early or delayed puberty, helping to discriminate between normal and abnormal GH status in short children.[27] However, there is no consensus on its use in clinical practice.[28]

We used the same peak GH cut-off for the ITT and glucagon tests. Adult studies have reported similar peaks in both tests.[29] GH responses to stimulation are a continuum and are poorly reproducible.[30] Although our locally derived cut-off (5 µg/L) is lower than the commonly used value of 7 µg/L, many studies have questioned the validity of this arbitrary value,[31] different assays may give widely differing results for GH,[32] and responses to the ITT may depend on how the test is performed.[33] Hence, GH cut-offs should be defined for each assay, laboratory and local reference population.[30] Niederland *et al*.[8] diagnosed GHD in 42% (11/26) of childhood TBI survivors using the 7 µg/L cut-off for the ITT and L-dopa tests but, as in our study, found no significant difference in height SDS between subjects with normal and abnormal responses.

Prolactin deficiency, observed in one subject with a borderline suboptimal cortisol response to the ITT but no other pituitary abnormality, is of little clinical significance in males. Often associated with severe hypopituitarism, it has been reported in one other TBI study.[34]

In contrast to two other paediatric studies,[7,9] we evaluated pituitary function fully in all our subjects. Poomthavorn *et al*. detected hormone abnormalities in 17% of severe TBI survivors but only half underwent baseline testing and only 8/54, stimulation testing.[7] Selection bias may have been increased by including results from four children found to have hypopituitarism before the study began. Einaudi *et al*. only performed stimulation tests in subjects with height velocities below the 25th percentile or low basal cortisol levels.[9]

In our study, we found no significant differences between normal and abnormal endocrine groups in terms of age at injury, age at study, or TBI severity. To date, associations between TBI severity and hypopituitarism observed in some cross-sectional studies[34] have not been substantiated in prospective[35] or paediatric studies.[8,9] We were unable to substantiate any link between the head injury characteristics evaluated and endocrine outcome. Other studies have identified basal skull fracture, diffuse axonal injury, raised intracranial pressure and prolonged intensive care admissions as risk factors for post-traumatic hypopituitarism.[3]

This study has some limitations. We recruited only 25% of all eligible subjects, a rate comparable with other retrospective paediatric TBI studies.[7,8,9] Relatively few TBI survivors with severe disability were recruited. Whilst the sample recruited was grossly representative of the wider cohort, recruited families were presumably more motivated to seek evaluation than non-responders. Conversely, parents of children with residual disability following TBI might have been reluctant to volunteer them for the study. Excluding subjects with small extradural or subdural haemorrhages, GCS scores of 13-15 and no requirement for ventilation or neurosurgery on ethical grounds could have introduced further selection bias. Although post-traumatic hypopituitarism has been described in children following minor TBI,[2,8,9] we could not be sure that the benefits of stimulation testing outweighed the risks in this group. A further limitation of the study is that height velocity was only evaluated in subjects with suboptimal GH responses, although height SDS gave no cause for concern in any subject. We considered that it was neither necessary nor practical to recall subjects with normal GH responses and normal height SDS for follow-up height measurements owing to their scattered geographical distribution.

In conclusion, no clinically significant endocrinopathy was identified amongst survivors of accidental childhood TBI evaluated at one to eight years post-injury, although minor abnormalities of the pituitary axes were observed. Growth and development should be monitored in survivors of childhood TBI but we do not consider that our findings justify

routine stimulation testing of the HPA and GH axes. However, the natural history of pituitary dysfunction occurring after childhood TBI needs further evaluation. Prospective longitudinal studies are required, although retention of subjects may be challenging.[9] Critical evaluation and interpretation of results, where possible in relation to (locally) validated assays and age- and pubertal stage-related reference ranges, is important in determining the likely clinical significance of any 'abnormalities' detected.

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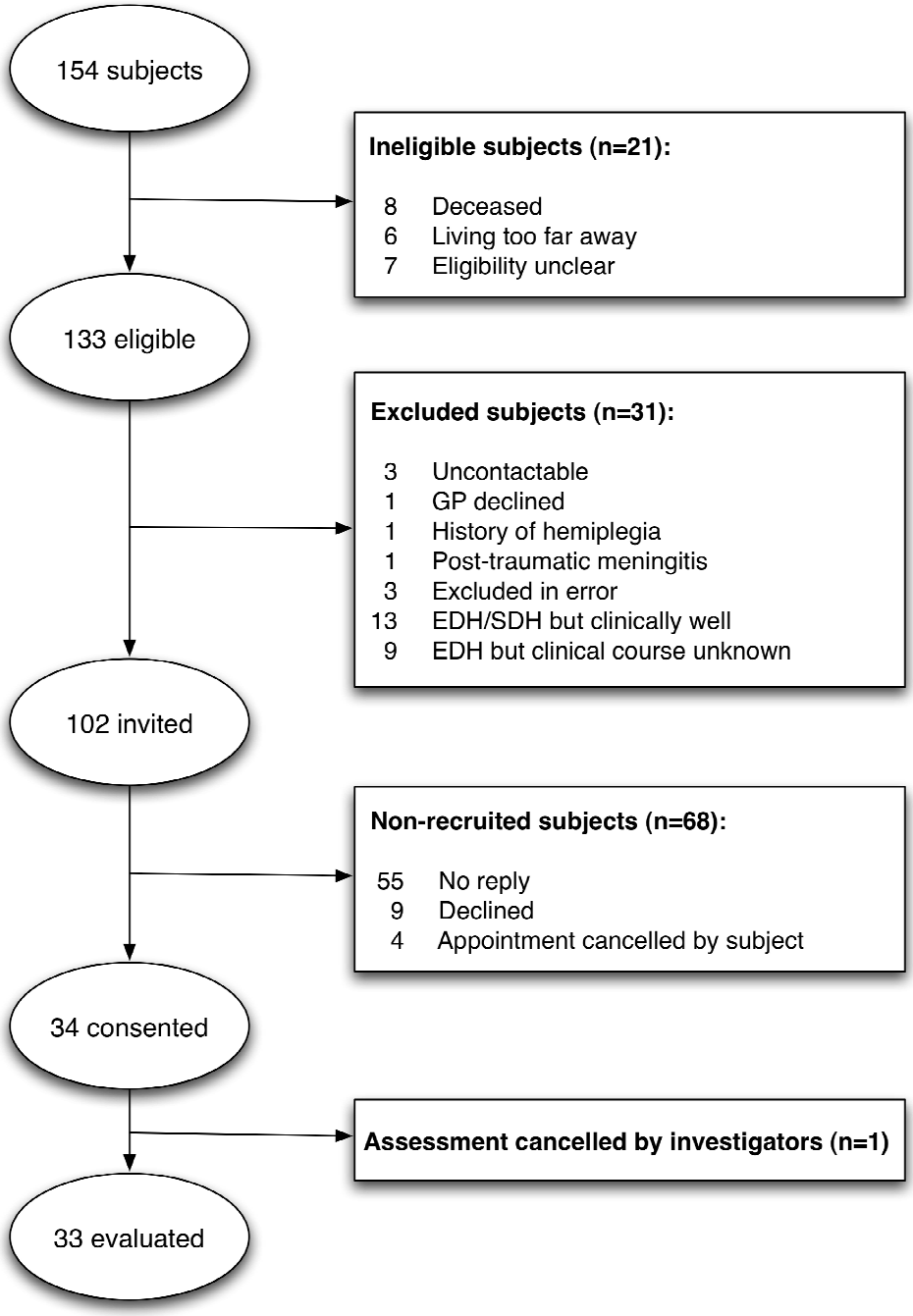
Figure 1: Flow diagram of subject recruitment



GP, general practitioner; EDH, extradural haemorrhage; SDH, subdural haemorrhage

Figure 2A: Cortisol responses to stimulation testing

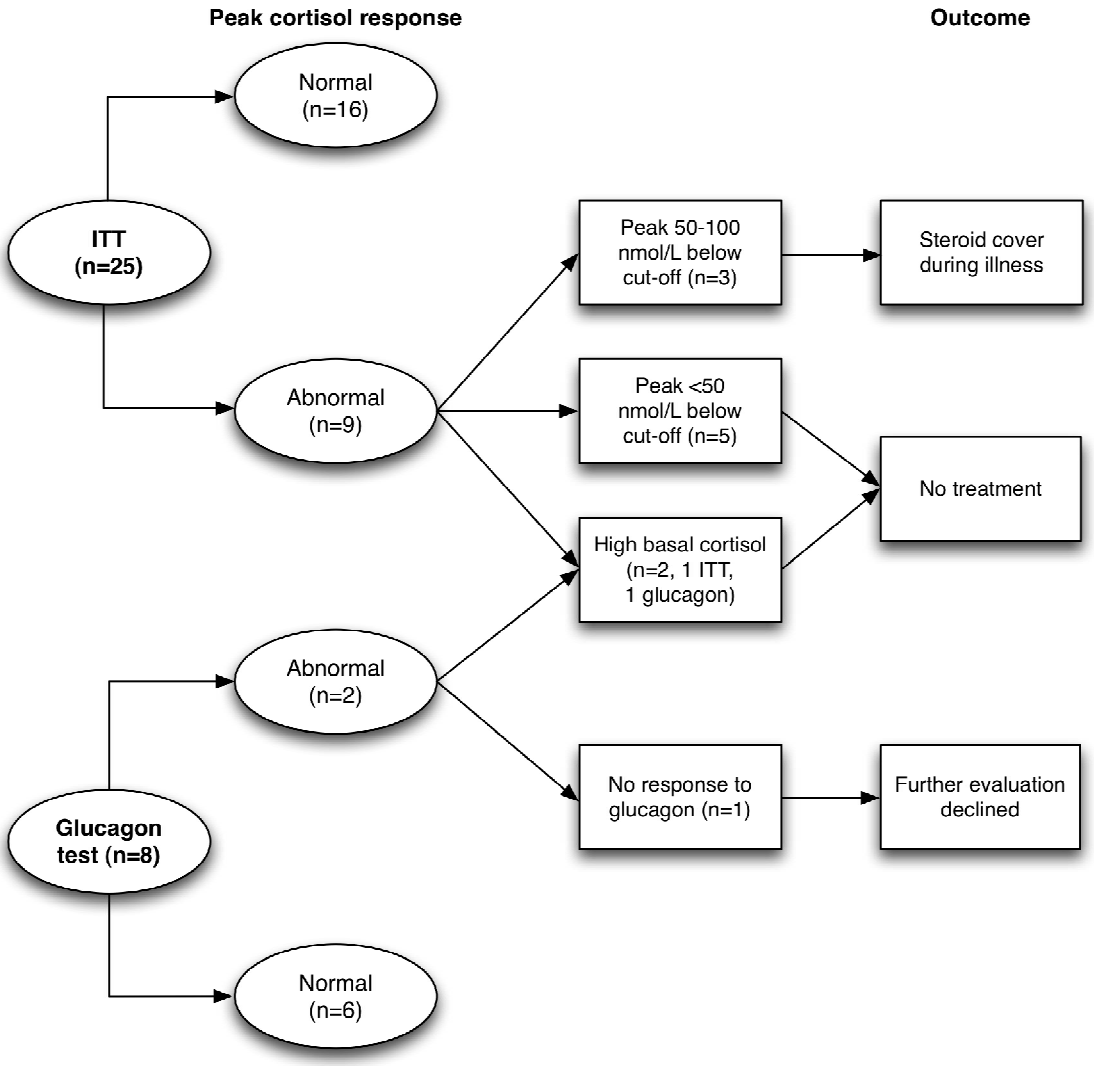


Figure 2B: GH responses to stimulation testing

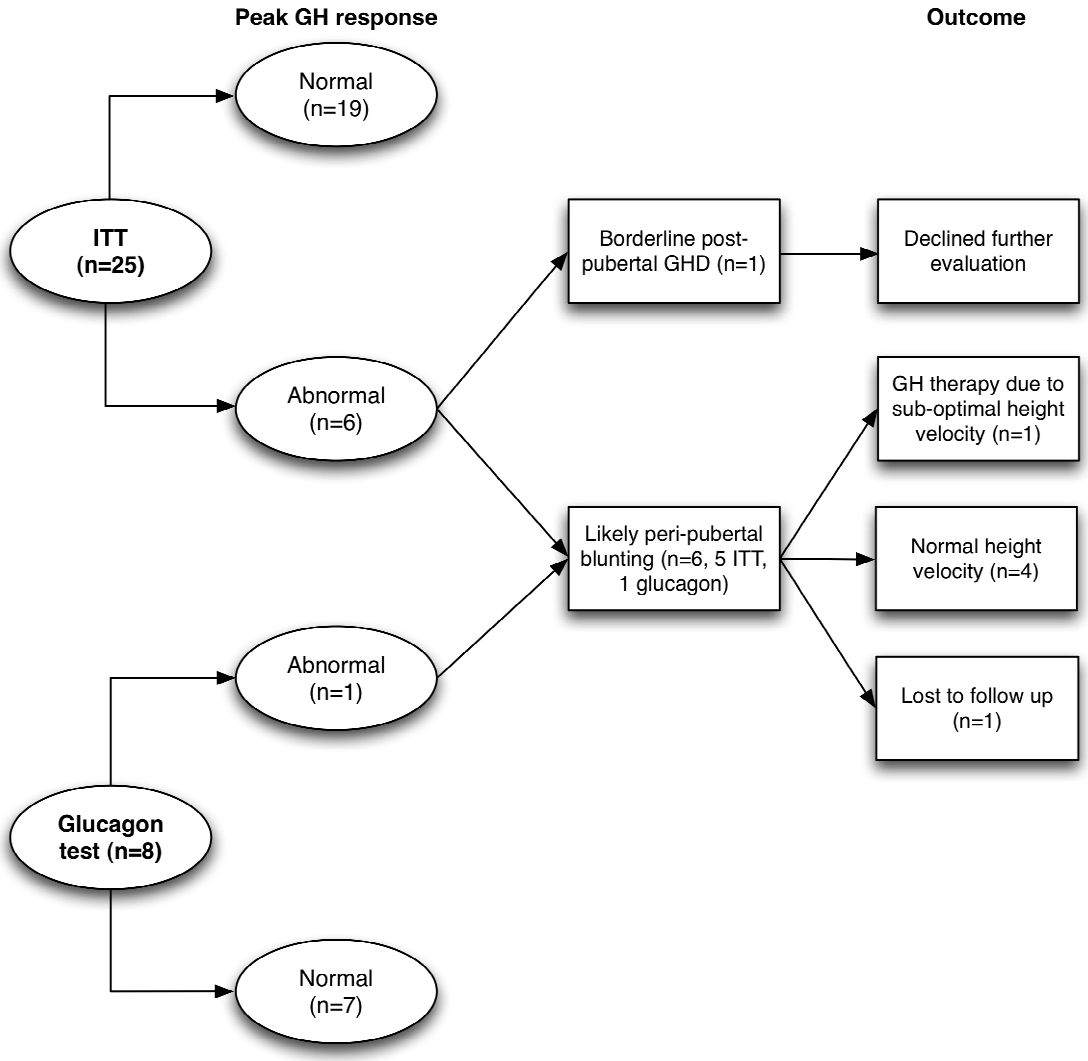


Table 1: Subject characteristics (n=33)

Characteristic	N, or mean/median* (range)
Gender (male / female)	25 / 8
Age at study (years)	13.4 (5.4–21.7)
Age at injury (years)	9.2 (0.6–14.1)
Interval since injury (years)	4.1 (1.4–7.8)
TBI severity (minor / moderate / severe)	6 / 15 / 12
KOSCHI score at study (good outcome / moderate disability / severe disability)	15 / 16 / 2
Pubertal status at study (pre- / early or mid- / late or post-)	10 / 7 / 16
Height SDS at study**	0.50 (-1.57 – +3.00)
Weight SDS at study**	0.65 (-1.28 – +2.86)
BMI SDS at study**	0.67 (-1.01 – +2.92)

*All mean (range) except for interval since injury, which is expressed as median (range)
**Excluding data for one subject on supra-physiological corticosteroid therapy.

Table 2: Subjects with abnormal pituitary hormone results

No.	Sex	TBI severity	Age (years) at study	Height SDS	Pubertal stage	GH/HPA stimulation test used	Hormone axis affected
1	M	Severe	16.4	0.64	Late/post-	ITT	HPA [1]
2	M	Severe	17.2	0.56	Late/post-	ITT	HPA [1]
3	M	Severe	17.0	-0.93	Late/post-	ITT	GH
4	M	Moderate	9.8	1.38	Pre-	Glucagon	GH
5	M	Moderate	9.0	0.10	Pre-	ITT	PRL & HPA [1]
6	M	Moderate	12.0	1.30	Early/mid-	ITT	GH & HPA [2]
7	M	Moderate	11.1	0.75	Pre-	ITT	GH
8	M	Moderate	15.1	0.90	Late/post-	Glucagon	HPA
9	M	Moderate	14.8	-1.52	Early/mid-	ITT	GH & HPA [1,3]
10	M	Moderate	12.9	1.00	Early/mid-	ITT	GH & HPA [2]
11	M	Minor	12.9	0.23	Early/mid-	ITT	HPA [1]
12	M	Minor	8.5	-0.31	Pre-	ITT	GH
13	F	Moderate	15.3	0.66	Late/post-	ITT	HPA [2,3]

M, male; F, female; PRL, prolactin; [1] peak cortisol response to ITT 0-50 nmol/L below cut-off; [2] peak cortisol response to ITT 50-100 nmol/L below cut-off; [3] low basal cortisol concentrations. See Methods for definition of cut-offs.