Salivary Steroid Profiles in the Assessment of Adrenocortical Function in Childhood Cancer Survivors

John Schulga MBChB, MRCGP, MRCP(I)

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University of Edinburgh

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Declaration

I acknowledge that solely the author, with the exception of areas of acknowledged collaboration, performed the work presented here. I performed the majority of the laboratory work, including the experiments required for the cortisol assay development. I also personally adapted the available assays for androstenedione and 17-OHP for use with saliva.

I performed all the field work, collected saliva samples form the control subjects and analysed all the control saliva samples as well as the study patient samples as well as analysing all the study patient blood spot samples.

I also performed all the low dose ACTH tests in the study patients.

Abstract

Children who are unfortunate enough to develop cancer are now able to achieve long-term survival as a consequence of improved treatment. However, a significant proportion of these children experience a high level of morbidity and their quality of life is of increasing concern. This morbidity relates not only to the primary disease, but also to the effects of the treatment they have received. An important consequence of radiotherapy and chemotherapy is the long-term effect on the endocrine system. Endocrine deficits can occur as a result of target organ damage to the thyroid, gonad or adrenal gland by both radiotherapy and chemotherapy, as well as damage to the hypothalamo-pituitary (HP) axis. While most endocrine deficit can be pre-empted by regular surveillance, the detection of secondary adrenocortical insufficiency (SAI) poses a particular problem. The symptoms of SAI (tiredness, lethargy, etc.) are non-specific, and are very similar to those that can result from anti-cancer therapy. Moreover, existing tests of adrenal function are difficult to interpret, and may not be diagnostic in children with mild to moderate degrees of SAI. It is in the child's interest for SAI to be promptly diagnosed and steroid replacement instigated. Correct diagnosis is essential as steroid therapy can have an adverse effect on growth and may cause weight gain.

This thesis aims to address the challenge of diagnosing SAI in long-term survivors of childhood cancer. The study group included patients who had received radiotherapy for brain tumours, total body irradiation as part of conditioning treatment for bone marrow transplantation, and patients with leukaemia who had relapsed and had received cranial irradiation as part of their treatment.

Sensitive assays of steroids in saliva (cortisol, 17-hydroxyprogesterone, and androstenedione) have been developed using radioimmunoassays with microencapsulated antibody. A reference range of these steroids in saliva has been determined using a control population of schoolchildren enabling salivary steroid profiles to be used in the assessment and monitoring of adrenocortical function in survivors of childhood cancer. These results have been compared with standard tests of adrenocortical function.

This thesis has shown that the use of salivary steroid profiles can be useful in the diagnosis of SAI. We have also shown that SAI is not as prevalent as has been reported in previous studies.

The methods developed in this study are simple and non-invasive and of clinical use in the ongoing assessment and monitoring of adrenal function in conjunction with established methods of assessing adrenal reserve.

Abbreviations

17-OHP	-	17-hydroxyprogesterone
ACTH	-	Adrenocorticotrophic hormone
ALL	-	Acute lymphoblastic leukaemia
AML	-	Acute myeloid leukaemia
AVP	-	Arginine vasopressin
BMT	-	Bone marrow transplantation
CAH	-	Congenital adrenal hyperplasia
cGy	-	Centigray
CNS	-	Central nervous system
CRH	-	Corticotrophin releasing hormone
DHEA	-	Dehydroxyepiandrosterone
DHEAS	-	Dehydroxyepiandrosterone sulphate
DNA	-	Deoxyribonucleic acid
GH	-	Growth hormone
GHRH	-	Growth hormone releasing hormone
GnRH	-	Gonadotrophin releasing hormone
Gy	-	Gray (unit of radiotherapy dose)
HP	-	Hypothalamo-pituitary
HPA	-	Hypothalamo-pituitary-adrenal
IGF-1	-	Insulin-like growth factor 1
ITT	-	Insulin tolerance test
LHRH	-	Leutinising hormone releasing hormone
PAM	-	Permeable antibody containing microcapsules
POMC	-	Pro-opiomelanocortin
RHSC	-	Royal hospital for sick children
RIA	-	Radioimmunoassay
SAI	-	Secondary adrenocortical insufficiency
SHBG	-	Sex hormone binding globulin
TBI	-	Total body irradiation
TRH	-	Thyroid releasing hormone
TSH	-	Thyroid stimulating hormone
UK	-	United Kingdom
UKCCSG	-	United Kingdom Childhood Cancer Surveillance Group

1 The Effects of Treatment in the Development of Endocrine Dysfunction in Childhood Cancer Survivors

1.1 Introduction

Children who are unfortunate enough to develop cancer are now able to achieve long-term survival. However, with prolonged survival of these patients, late complications of their treatment are a recognised cause of morbidity and their quality of life is of increasing concern. Morbidity in these long-term survivors relates not only to the primary disease itself, but also to the effects of the various types of treatment (Byrd 1983; Deeg et al 1984; Ochs & Mulhern 1988; Sklar & Nesbit 1981). An important consequence of chemotherapy, and in particular, radiotherapy, is the long-term effect on the endocrine system. There are numerous reports and studies in the current literature of various endocrine deficits that occur as a result of treatment of childhood cancers (Brown et al 1983; Oberfield et al 1986; Ogilvy-Stuart et al 1992; Ogilvy-Stuart et al 1994; Oliff et al 1979; Sanders et al 1986; Shalet et al 1975; Shalet et al 1976a; Shalet et al 1988).

Endocrine deficits can occur as a result of target organ damage to the thyroid, gonad or adrenal gland by both radiotherapy and chemotherapy. (Leiper et al 1987a; Littley et al 1991; Oberfield, Allen, Pollack, New, & Levine 1986; Ogilvy-Stuart, Clark, Wallace, Gibson, Stevens, Shalet, & Donaldson 1992). In addition, they may result from damage to the hypothalamo-pituitary (HP) axis (Brown, Lee,

Eden, Bullimore, & Savage 1983; Littley et al 1989; Oberfield, Allen, Pollack, New, & Levine 1986; Ogilvy-Stuart, Clayton, & Shalet 1994; Shalet, Beardwell, Morris-Jones, & Pearson 1975; Shalet et al 1976b; Shalet, Clayton, & Price 1988). The original disease process may also cause endocrine deficit, depending on the anatomical site (e.g. a hypothalamic tumour, or a tumour encroaching on the HP territory).

While most endocrine deficits can be pre-empted by regular surveillance, the detection of secondary adrenocortical insufficiency (SAI) poses a particular problem. This is because the symptoms of SAI (tiredness, lethargy, etc.) are non-specific, and may be difficult to differentiate from symptoms which can occur as a consequence of anti-cancer therapy. Moreover, existing tests of adrenal function are difficult to interpret, and may not be diagnostic in children with mild to moderate degrees of adrenocortical insufficiency. For example, in SAI, the cortisol response to standard adrenocorticotrophic hormone (ACTH) stimulation may be normal (Cunningham et al 1983).

Incomplete or partial SAI may result in years of poor general health with lack of energy especially on rising, tiredness, lethargy, and difficulty in shaking off trivial inter-current illness (Leshin 1982; Mansell et al 1993). Clearly, it is in the child's interest for adrenocortical insufficiency to be promptly diagnosed and steroid replacement instigated. Correct diagnosis is essential as steroid therapy can have an adverse effect on growth and may cause weight gain. The unnecessary use of hydrocortisone in these patients should therefore be avoided.

This study attempts to address the problem of diagnosing SAI in long-term survivors of childhood cancer. In particular, the main aim is to develop and evaluate a sensitive method of diagnosing or screening for SAI in a manner that involves minimal hazard and upheaval to the child. If successful, such a method should

allow an accurate assessment of the incidence of SAI, and facilitate the monitoring of at-risk children.

Although relatively rare in childhood, cancer is a significant cause of death. By the age of 15 years approximately 1 child in 650 is affected by malignant disease (UKCCSG 1996) (Figure 1.1). Children can be affected by a variety of malignancies. Recent data demonstrate that over one third are leukaemias, and almost one quarter originates in brain and spinal cord (Stiller 1992) (Figure 1.2).

Cancer survival rates are improving. In a study based on figures obtained from the Manchester Children's Tumour Registry, survival rates in various childhood cancers were compared from three decades: 1954 - 1963, 1964 - 1973, and 1974 – 1983 (Birch et al 1988). For all cancers, there was a more than two-fold increase in survival (from 21 to 49%) between the first and third decades. There was a more than 23-fold improvement in leukaemia survival (2 to 47%), and a six-fold improvement in germ cell tumour survival. Medulloblastoma survival improved from 25% to 41%. Similar results have also been reported in other studies (Schellong 1985).

Improvements in cancer treatment have increased the survival rates for the majority of childhood cancers, especially leukaemias (Figure 1.3).

Survival rates in childhood cancer depend on the type and the extent of disease at presentation; this in turn determines the treatment regimen for the particular child. The improved survival in the leukaemias has been largely the result of the development of effective chemotherapy protocols using multiple agents. Improved radiotherapy techniques for solid tumours, has helped to minimise the development of side effects, with maximal tumour resolution. Some of the improvements in survival can be related to centralisation of treatments for all cancers in specialist centres (Stiller 1988).

What constitutes cure for childhood cancer? In the Manchester study, only 1% of patients died from recurrence more than 10 years after diagnosis. Disease free survival 10 years from treatment may therefore be regarded as "cure" of the primary cancer. With current survival figures, it has been estimated that by the year 2000, 1 in 1000 children will be survivors of childhood cancer (Birch, Marsden, Morris-Jones, Pearson, & Blair 1988).

With the use of powerful chemotherapeutic agents and improved radiotherapy, the price of survival may be an increase in the prevalence of late effects of cancer therapy, including endocrine, psychological, intellectual, cardiac and renal effects. Endocrine late effects have been related to radiotherapy particularly affecting the HP region, the thyroid, and gonads, and may not become manifest for years after treatment. It was believed that the site of radiation damage was the pituitary gland, but more recent evidence indicates that it is the hypothalamus that is principally affected (Stiller 1992). Chemotherapy is increasingly recognised as having an important role in predisposing to endocrine late-effects.

Most endocrine late effects relate to the treatment of leukaemias and brain tumours that has involved radiotherapy and/or chemotherapy to the HP axes (growth, gonads, thyroid and adrenal).

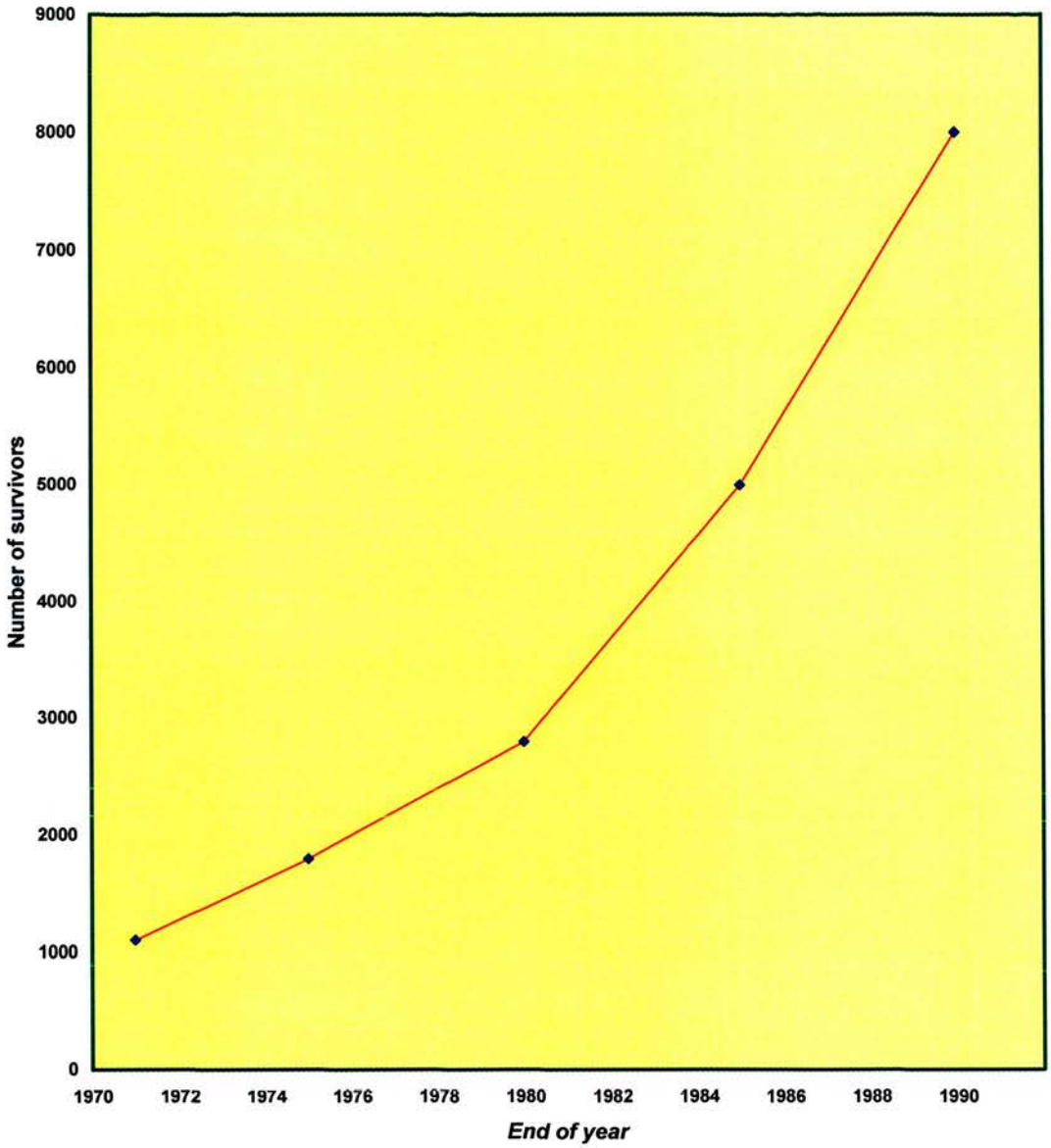

Figure 1.1. Number of adults in the UK who have survived childhood cancer (1971 - 1990, UKCCSG)

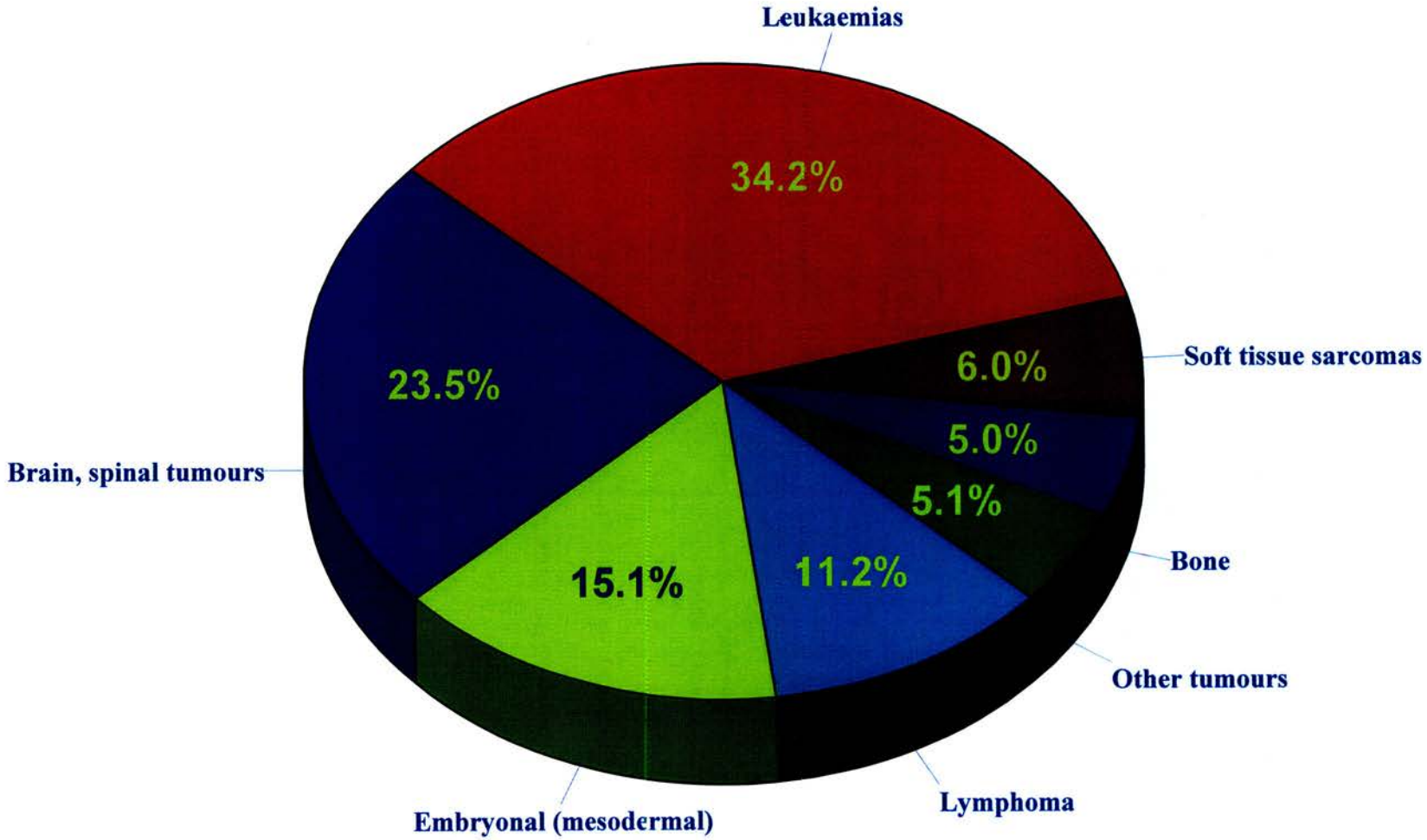

Figure 1.2 Prevalence of Childhood Cancers (UKCCSG Figures 1990)

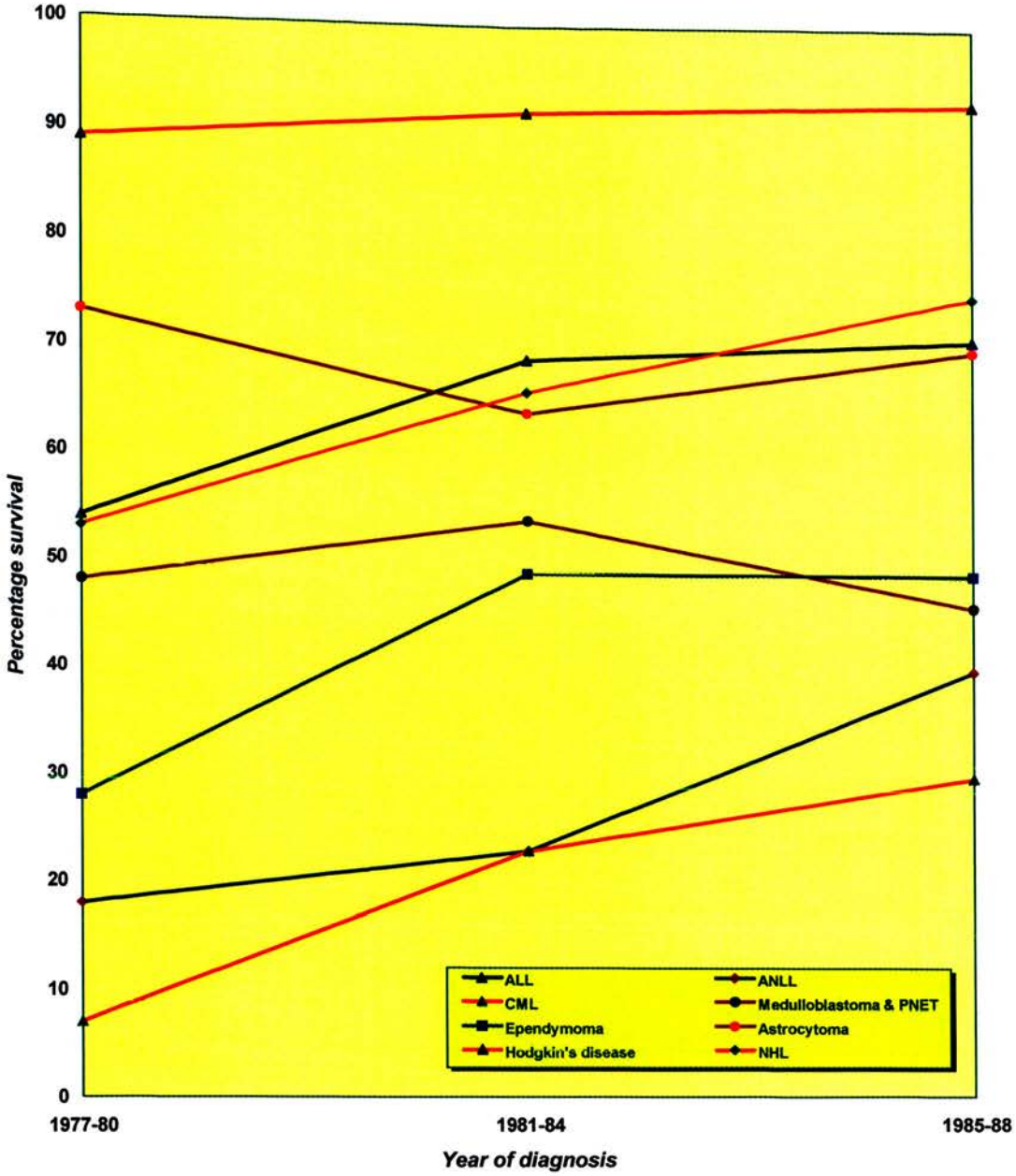

Figure 1.3. Five year survival rates for childhood cancer survivors, 1977 to 1988 (UKCCSG figures)

1.1.1 The Childhood Leukaemias: Effects on Growth and Endocrine Function

The treatment of childhood leukaemia has been continually modified and improved to reduce adverse effects and improve the chances of survival. The Medical Research Council has in the context of formal clinical trials established treatment for most children in the United Kingdom (UK) (UKCCSG 1995).

Survival from Acute lymphoblastic leukaemia (ALL) has increased dramatically over the past thirty years (Birch, Marsden, Morris-Jones, Pearson, & Blair 1988) (Figure 1.3). A study in 1989 reported an overall first remission rate of 94%, and an event free survival rate at 8 years of 44% (Van Eys et al 1989). These figures have been echoed in other studies (Birch, Marsden, Morris-Jones, Pearson, & Blair 1988).

1.1.1.1 Chemotherapy and Cranial Prophylaxis

ALL treatment uses certain chemotherapeutic agents in carefully calculated combinations. They have included maintenance treatment with 6-mercaptopurine and methotrexate with regular pulses of prednisolone, vincristine and daunorubicin, as well as pulses of cytarabine, etoposide, thioguanine and asparaginase. From 1972, low dose cranial irradiation (18 to 24 Gy) has been used in all protocols as prophylaxis against central nervous system involvement with leukaemia (Aur et al 1972). The use of cranial irradiation has greatly improved the prognosis of children treated for ALL (Hustu et al 1973). More recently, in an attempt to reduce the cognitive, psychological and growth sequelae of cranial irradiation, particularly in the younger child, the MRC UKALL XI protocol has been instituted to assess the benefits of either high dose intravenous or intrathecal methotrexate in preference to cranial irradiation (Byrd 1983; Ochs & Mulhern 1988). This protocol resulted from evidence presented by the American Children's Cancer Study Group that showed

that children in a 'good risk' group could be treated without cranial irradiation if long-term intrathecal methotrexate was given (American CCSG 1982; Littman et al 1987).

Endocrine deficits in ALL include disturbance of GH secretion, onset of early puberty, body disproportion and altered body composition. Factors causing these problems include cranial irradiation, chemotherapy, and preparation for bone marrow transplantation. The most important cause of endocrine deficit is cranial irradiation. In general, a lower dose of radiotherapy is employed in cranial prophylaxis for ALL in order to prevent central nervous system (CNS) seeding, compared with radiotherapy of brain tumours, in which much higher doses are used.

1.1.1.1.1 Growth Hormone deficiency

In children treated with cranial irradiation, one of the most important late effects is that on growth and growth hormone. It has been estimated that 25 to 50% of ALL survivors have reduced GH secretion; moreover, the predicted final height for these children is seldom attained (Byrd 1983; Ochs & Mulhern 1988; Oliff, Bode, Bercu, Di Chiro, Graves, & Poplack 1979; Romshe et al 1984). Understandably, this is the most frequently studied abnormality of endocrine function following cancer treatment. Several studies have produced conflicting results of effects on growth as a result of this 'low dose' radiotherapy. In 1988, a study by Clayton et al showed that there was a reduction in growth in children who had received 1800 to 2500cGY cranial irradiation, up to four years after treatment (Clayton et al 1988). This was also found by other workers (Starceski et al 1987; Wells et al 1983). However, Shalet et al found that children treated for ALL with cranial or craniospinal irradiation (2400 or 2500cGy) were significantly smaller than a control group, and had a normal growth pattern despite showing a diminished growth hormone response to arginine and hypoglycaemia (Shalet et al 1979). Other workers have also shown a normal growth pattern in this group of children, but GH secretion was either normal or

abnormal (Shalet et al 1977; Swift et al 1978; Verzossa et al 1976). In 1986, the results of a study was reported in which 4 out of 14 children in remission of their ALL for at least 3 years had biochemical evidence of GH deficiency (Asa et al 1986). None of the children had any other detectable endocrine abnormality.

In 1984, reduced pulsatile GH secretion was shown to occur over 24 hours in 8 long-term ALL survivors who were treated with 2400cGy cranial irradiation 4 to 10 years previously (Blatt et al 1984). The area under the curve for growth hormone profiles was reduced in these patients compared to a control population, as was growth hormone output through the night and during the day. The frequency and amplitude of growth hormone pulses were also reduced. In this group of patients, 3 had a normal GH response to hypoglycaemia, two had a borderline response.

In 1977, in a study of 77 children with ALL treated with chemotherapy and 2400 cGy cranial irradiation (given in 15 fractions over 3 weeks) 3 to 9.5 years previously, growth was shown to be reduced, demonstrated by a reduction in the standard deviation score over time from diagnosis (Kirk et al 1987). A much higher prevalence of growth hormone deficiency was found (30 of 42 children tested) than had been reported in other studies. Pulsatile growth hormone secretion was also reduced.

1.1.1.1.2 Thyroid Dysfunction

Abnormalities of thyroid function are well described in children treated with cranial or craniospinal irradiation for ALL. There is a very low risk of developing thyroid deficiency, with a reported prevalence of 0.2% (Robison et al 1985). In this study, however, 10% of children had a raised TSH level with normal thyroxine levels suggestive of a compensated euthyroid state. The majority of these children had subsequent normal TSH concentrations. All of them were clinically euthyroid.

1.1.1.1.3 Gonadal and Pubertal Dysfunction

Pubertal dysfunction, in particular, early or precocious puberty, is a recognised consequence of low dose cranial irradiation in ALL prophylaxis. It is associated with a high prevalence of earlier age of pubertal onset and menarche in girls (Leiper, Stanhope, & Kitching 1987a; Quigley et al 1989). Early puberty has also been documented in boys surviving ALL, although the prevalence is similar to the normal population.

1.1.1.2 Bone Marrow Transplantation

Bone marrow transplantation (BMT) in combination with high dose chemotherapy is used with success in an increasing number of children with malignant haematological conditions such as ALL and acute myeloblastic leukaemia (AML). BMT is also used in the treatment of stage IV neuroblastoma. BMT preparatory regimens include the use of chemotherapeutic agents such as cyclophosphamide and cytosine arabinoside, together with total body irradiation (TBI) in the treatment of malignant disease. In non-malignant conditions BMT is a successful method of treatment used without TBI. The TBI dose has in the past been given as a single dose, or in a dose, which is fractionated. Latest regimens now use only fractionated TBI doses, as single dose TBI results in a greater incidence of side effects (Brauner et al 1993; Sanders, Pritchard, Mahoney, Amos, Buckner, Witherspoon, Deeg, Doney, Sullivan, Appelbaum, Storb, & Thomas 1986). Children with non-malignant disease, such as aplastic anaemia, are also treated with BMT, but TBI and chemotherapy are not used in BMT preparation.

BMT preparative regimens, particularly the use of single dose TBI, are a potent cause of endocrine dysfunction (Deeg, Storb, & Thomas 1984). Pituitary dysfunction is well recognised, with a high incidence of GH deficiency (up to 70% of survivors) (Sanders, Pritchard, Mahoney, Amos, Buckner, Witherspoon, Deeg,

Doney, Sullivan, Appelbaum, Storb, & Thomas 1986). There is also a high incidence of primary thyroid dysfunction (39%) and primary gonadal failure (68%). In postpubertal children, gonadal failure was uniformly observed. The likelihood of gonadal and thyroid dysfunction was not related to previous cranial irradiation in children who had received single dose TBI (Leiper et al 1987b).

Fractionated TBI has a lower incidence of GH deficiency in adults (Littley, Shalet, Morgenstern, & Deakin 1991) but similar rates of thyroid dysfunction. Other studies have confirmed a high incidence of GH deficiency together with gonadal and thyroid dysfunction in children (Papadimitriou et al 1991). However, GH deficiency is felt by some researchers to be the result of previous cranial irradiation; the total dose of cranial irradiation received is therefore important (Brauner, Fontoura, Zucker, Devergie, Souberbielle, Prevot-Saucet, Michon, Gluckman, Griscelli, Fischer, & Rappaport 1993). Other studies have not been able to substantiate this finding (Shalet, Clayton, & Price 1988).

Fractionated TBI is now known to produce fewer endocrine deficits, although the endocrine morbidity it causes still remains significant (Ogilvy-Stuart, Clark, Wallace, Gibson, Stevens, Shalet, & Donaldson 1992).

1.1.2 Brain Tumours: Effects on Growth and Endocrine Function

Intracranial tumours are the second most common malignancy of childhood, after leukaemia. They comprise 25% of childhood malignancies (Birch, Marsden, Morris-Jones, Pearson, & Blair 1988) (Figure 1.2). Up to 25% of brain tumours are medulloblastomas. The five-year survival rate for brain tumour patients is continuing to improve, but remains relatively low; for patients with medulloblastoma the survival rate is approximately 50% (UKCCSG Figures).

Treatment of brain tumours in children generally consists of high doses of cranial irradiation (commonly in excess of 35 Gy) together with surgery, and occasionally chemotherapy. Cranial irradiation involves treatment to the whole brain, as well as to the tumour site. In the treatment of medulloblastoma, the spinal cord is also irradiated.

Despite improvements in survival for these children, the late effects of treatment are high, especially the endocrine deficits (Brown, Lee, Eden, Bullimore, & Savage 1983; Oberfield, Allen, Pollack, New, & Levine 1986). GH deficiency is common (Brown, Lee, Eden, Bullimore, & Savage 1983; Shalet, Beardwell, Morris-Jones, & Pearson 1975; Shalet, Beardwell, Pearson, & Morris-Jones 1976b; Sklar & Nesbit 1981; Woodside et al 1991). In general, the higher the dose of irradiation the more likely and the earlier the onset of growth hormone deficiency.

In 1975, Shalet et al found an abnormal growth hormone response to hypoglycaemia in 10 out of 27 children treated for intracranial tumours out with the HP axis (Shalet, Beardwell, Morris-Jones, & Pearson 1975). The TSH response to TRH was also abnormal in the growth hormone deficient children. In 1976, the same group subsequently found that there was a significant inverse correlation between radiation dose and peak growth hormone response to hypoglycaemia in children treated with high dose cranial irradiation (greater than 2900cGy) for brain tumours out with the HP axis (Shalet, Beardwell, Pearson, & Morris-Jones 1976b). It was calculated that the threshold dose for growth hormone deficiency in these children was 2900cGy, although data was not available in the dose range 2500 - 2900cGy. In 1986, evidence was presented which showed that GH deficiency in children occurred if they were treated with at least 2400cGy cranial irradiation (Ahmed et al 1986).

A retrospective study by Winter et al in 1985 (Winter & Green 1985) found that in 5 patients treated with high dose cranial irradiation who were growth hormone deficient, the growth response to growth hormone therapy was blunted and skeletal maturation was augmented. However, some of the children had had treatment for pituitary-hypothalamic lesions, which themselves may interfere with growth hormone secretion.

In 1983, a study found abnormal growth in all 13 children with medulloblastoma who were studied (Brown, Lee, Eden, Bullimore, & Savage 1983). Nine patients had an abnormal GH response to hypoglycaemia. In a similar study, 14 out of 19 children with medulloblastoma had decreased growth rates, but 5 children had abnormal GH response (Oberfield, Allen, Pollack, New, & Levine 1986).

In addition to growth hormone deficiency, thyroid and pubertal dysfunction are well documented. In medulloblastoma patients treated with high dose cranial irradiation, a high prevalence of elevated TSH levels were found, with a high incidence of compensated hypothyroidism in these patients (Oberfield, Allen, Pollack, New, & Levine 1986). One case of each of primary and tertiary hypothyroidism was found in the group of 22 patients studied.

Onset of puberty occurs at an earlier age in both treated girls and boys (Brauner et al 1984; Brauner & Rappaport 1990; Moris 1981). The younger the child at irradiation, the earlier the age of onset of puberty (Ogilvy-Stuart, Clayton, & Shalet 1994).

1.1.3 Radiotherapy and its Effects on the Hypothalamo-Pituitary Axis

Cranial irradiation is an important part of treatment of childhood malignancy. The dose used, the number of fractions, the radiation field and the lengths of treatment depend on the nature of the initial disease and the reason for cranial irradiation (e.g.

prophylaxis or tumour destruction). These factors in turn influence the likelihood of late endocrine effects resulting from anterior pituitary hormone deficit. (see Table 1.1, 1.2) The magnitude of dose of radiotherapy given is dependent on the type of cancer (see Table 1.3). The degree of anterior pituitary hormone deficit is related to the radiation dose received by the hypothalamo-pituitary axis, higher doses producing panhypopituitarism, whereas lower doses cause isolated GH deficiency (Shalet, Clayton, & Price 1988).

In 1989, a Manchester group (Littley, Shalet, Beardwell, Ahmed, Applegate, & Sutton 1989) studied the development of anterior pituitary deficiencies in adults treated with radiotherapy for pituitary tumours. The majority had had surgery prior to radiotherapy.

A high incidence of anterior pituitary hormone deficiencies were found in this group, with all patients growth hormone deficient after 5 years, 91% gonadotrophin deficient, 77% corticotrophin deficient, and 42% thyrotrophin deficient. The prevalence increased with time (Figure 1.4). The sequence of development of hormone deficiencies was found to occur most commonly in that order. This study has been used by many workers to state the rate of onset of endocrine deficiencies in children who had received cranial irradiation but who did not have intrinsic pituitary disease.

The effects of varying doses of cranial irradiation on endocrine function in children have been studied by several groups who have tended to study effects on growth hormone production. The development of endocrine deficit is related not only to the dose received but also to the interval. Thus, Jenkins et al reported that radiotherapy in doses of 3.5 to 5.0 Gy in patients with pituitary tumours was generally well tolerated with little effect on residual pituitary function (Jenkins et al 1972).

Romshe et al studied a group of 9 children who had received cranial irradiation of at least 1500 cGy 2 years or more prior to commencement of the study (Romshe, Zipf, Miser, Miser, Sotos, & Newton 1984). They found significant GH deficiency in this group, with abnormal growth hormone response to hypoglycaemia, and abnormal GH profile of GH pulsatility, but a normal response to arginine and L-dopa. However, the patient group was heterogeneous, with two patients treated for medulloblastoma.

Cranial irradiation causes damage to the hypothalamus and the pituitary with the hypothalamus being more vulnerable. In 1982 a discordant response to arginine and insulin hypoglycaemia was demonstrated in the cranial irradiated monkey (Chrousos et al 1982). Chrousos also demonstrated severe blunting of physiological GH secretion despite a normal response to pharmacological stimulus. Similar results have been obtained in humans (Dickinson et al 1978; Shalet, Price, & Beardwell 1979). It has also been shown that despite a subnormal GH response to hypoglycaemia, patients responded normally to GHRH (Ahmed & Shalet 1984; Lannering & Albertsson-Wiklund 1987). A study in 1986 (Lam et al 1986) showed evidence of hypothalamic dysfunction in patients treated for nasopharyngeal carcinoma, with raised prolactin levels, normal response to GnRH with subnormal gonadal function, normal but delayed TSH response to TRH in the presence of subnormal thyroid function, and normal ACTH response to CRH in the presence of subnormal adrenocortical function.

Site of initial disease
Dose of Radiotherapy
Site of radiotherapy
Age at commencement of radiation treatment
Number of years from treatment
Chemotherapeutic agents used

Table 1.1. Factors affecting likelihood of developing endocrine deficit in children treated for cancer

Type of treatment	Radiation dose (Gy)	Endocrine deficit
Cranial irradiation	**18 to 24**	Growth hormone insufficiency; impaired growth
		Early puberty; Precocious puberty
Cranial irradiation	**>24**	Growth hormone deficiency
		Early puberty; Precocious puberty
		Gonadotrophin deficiency
		Adrenocorticotrophin deficiency
		Secondary hypothyroidism (uncommon)
Craniospinal irradiation	**>30**	Anterior pituitary hormone deficiencies
		Primary hypothyroidism
		Poor spinal growth
Testicular irradiation	**6**	Gonadal failure - Leydig cell damage
		Infertility
Total body irradiation	**12 to 14**	Primary hypothyroidism
		Primary gonadal failure (male and female)
		Growth hormone deficiency
Chemotherapy		Reduced bone growth
		Primary gonadal damage
		Compensated hypothyroidism

Table 1.2. The potential effect of chemotherapy and varying doses of radiotherapy on the development of endocrine deficit.

(Shalet et al 1979; Swift et al 1976; Asa et al 1986; Blatt et al 1984; Leiper et al 1987; Quigley et al 1989; Sanders et al 1986; Brown et al 1983; Shalet et al 1975; Sklar et al 1981; Woodside et al 1991; Ahmed et al 1986; Ogilvy-Stuart et al 1995)

Malignancy	Location of irradiation	Total dose to head (cGy)	Estimated total pituitary dose (cGy)
ALL initial presentation	Cranial/craniospinal	1800	1800
ALL CNS relapse	Cranial	600 - 2400	600 - 2400
BMT	Total Body	1200 - 1440	1200 - 1440
Medulloblastoma	Cranial Craniospinal Posterior fossa	3500 3300 1500	4800
Optic Glioma	Cranial	5000	5000
Germ cell Tumour	Craniospinal	3500	3500

Table 1.3. Standard irradiation doses used in the treatment of childhood malignancy

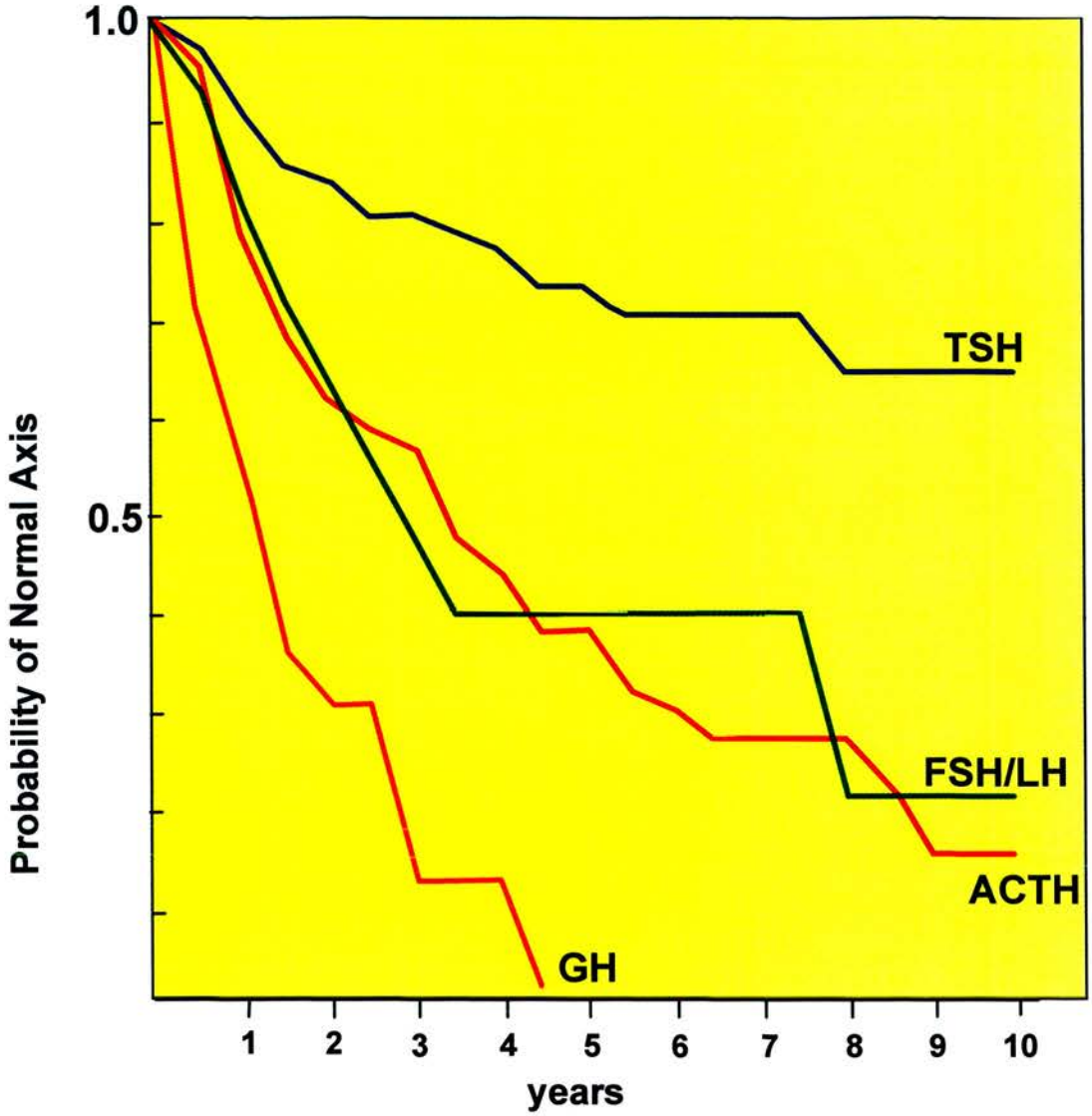

Figure 1.4. Likelihood of developing anterior pituitary hormone deficiencies with time. (Littley et al)

(Derived from adults with pituitary tumours who received between 37 to 42 Gy external radiotherapy in 15 or 16 fractions over 20 to 22 days).

1.1.4 Chemotherapy and its Effects on the Hypothalamo-Pituitary Axis

Endocrine dysfunction as a consequence of chemotherapy is difficult to ascertain, as the use of chemotherapy along with radiotherapy in malignant disease is universal. There is in-vitro evidence to suggest that chemotherapy affects the epiphyseal growth plate, (Moris 1981) but little conclusive evidence to state that chemotherapy has a definite effect in vivo.

Evidence is accumulating that there is a cumulative effect of both radiotherapy and chemotherapy on growth, and probably also on gonadal function (Ogilvy-Stuart & Shalet 1995).

2 Assessment of the Hypothalamo-Pituitary-Adrenocortical Axis

2.1 Overview of the Hypothalamo-Pituitary-Adrenal Axis

The hypothalamo-pituitary-adrenal (HPA) axis regulates the production of glucocorticoid, principally cortisol, and to a much lesser extent, mineralocorticoid, mainly aldosterone. Corticotrophin releasing hormone (CRH) is produced in the paraventricular nucleus of the hypothalamus and acts synergistically with arginine-vasopressin (AVP) to stimulate ACTH release from the corticotroph cells of the anterior pituitary gland. This in turn stimulates steroid synthesis and release from the adrenal cortex. Glucocorticoids released from the adrenal cortex have an important role to play in controlling the body's metabolic processes.

2.1.1 Control of Glucocorticoid Release

Harris and Green in 1947, proposed that hypothalamic release of a humoral signal occurred in response to stress (Green & Harris 1947). It was not until 1955 when Guillemin and Rosenberg, and Saffran and colleagues independently demonstrated CRH-like activity in hypothalamic extracts (Guillemin & Rosenberg 1955; Saffran et al 1955). This was the first hypophysiotrophic activity to be seen. The chemical identity of CRH was not established until 1981 (Vale et al 1981). Ovine CRH was subsequently synthesised and shown to stimulate pituitary ACTH secretion and release (Vale et al 1983).

Circadian ACTH secretion is believed to be controlled by several factors, including CRH and AVP stimulation. Light is thought to be one of the most important factors in controlling the circadian rhythm (Moore-Ede et al 1983; Orth et al 1979). However, stress-mediated release of ACTH may occur in synergism with AVP, CRH, catecholamines and other secretagogues whose release is initiated by the higher centres in the brain (Antoni 1986; Axelrod & Reisine 1984; Feek et al 1983; Rivier & Vale 1983). CRH neurones are in turn regulated by specific hypothalamic neurotransmitters such as serotonin, acetylcholine and noradrenaline. It is felt that these play a significant role in the control of both circadian and stress-mediated release of CRH (Calogero et al 1988; Jones et al 1976).

CRH is released from the hypothalamus into the portal circulation and stimulates the release of pro-opiomelanocortin (POMC) through an adenylate cyclase-mediated process. The control of CRH release is by a negative feedback loop with cortisol.

CRH stimulated release of ACTH is one of three regulatory systems for ACTH secretion; there is also a closed loop negative feedback system responding to cortisol, and an open-loop neuronal system channelling stress stimuli (Jones & Gillham 1988).

ACTH is the main regulator of adrenal glucocorticoid secretion and may be the main regulator of adrenal androgen secretion. However, an adrenal androgen-stimulating factor has been postulated but not yet isolated (Cunningham & McKenna 1994). ACTH seems to have a minor role in mineralocorticoid secretion.

ACTH is released in an episodic, pulsatile manner that results in a similar pulsatile release of cortisol. The normal diurnal rhythm results from pulsatile release of ACTH of varying amplitude. The pulses increase in amplitude, reaching a

maximum in the last few hours before and the hour after wakening. These then become smaller in amplitude throughout the day, becoming minimal in the evening (Moore-Ede, Czeisler, & Richardson 1983).

ACTH acts through specific high affinity cell membrane receptors in the adrenal cortex. ACTH activates adenylate cyclase, resulting in cyclic AMP accumulation in the adrenocortical cells, increasing protein kinase A activity. This results in cortisol synthesis and secretion (Simpson & Waterman 1988).

A number of factors affect CRH and ACTH release. The circadian rhythm is generated by an endogenous pacemaker located in the suprachiasmatic nucleus of the hypothalamus. The timing of the rhythm is synchronised with the normal daily pattern of darkness and light, which normally reflects the sleep-wake pattern. In the absence of darkness and light as cues (e.g. in blind people), the circadian rhythm has a 24.5 to 25 hour period (Orth, Besser, & King 1979). The exact mechanism for this circadian rhythm is unknown. It is felt that CRH has some role to play, but other factors are involved.

As well as the diurnal rhythm, which governs HPA function, stress (both physical and psychological) has a potent effect on stimulating the HPA axis. Stress is a poorly defined term, which includes severe events such as trauma, burns, illness, major surgery, hypotension and collapse, and everyday experiences like anxiety, anticipation and mental tasks. The way in which stress exerts its effects on ACTH secretion is unknown. It appears to act via a central pathway, stimulating the hypothalamus to release CRH and AVP, which in turn stimulate ACTH release. CRH may also be directly released by pyrogen, and possibly also by interleukins one and six released by endotoxin. Interleukin two may stimulate ACTH release both directly and indirectly (Bateman et al 1989).

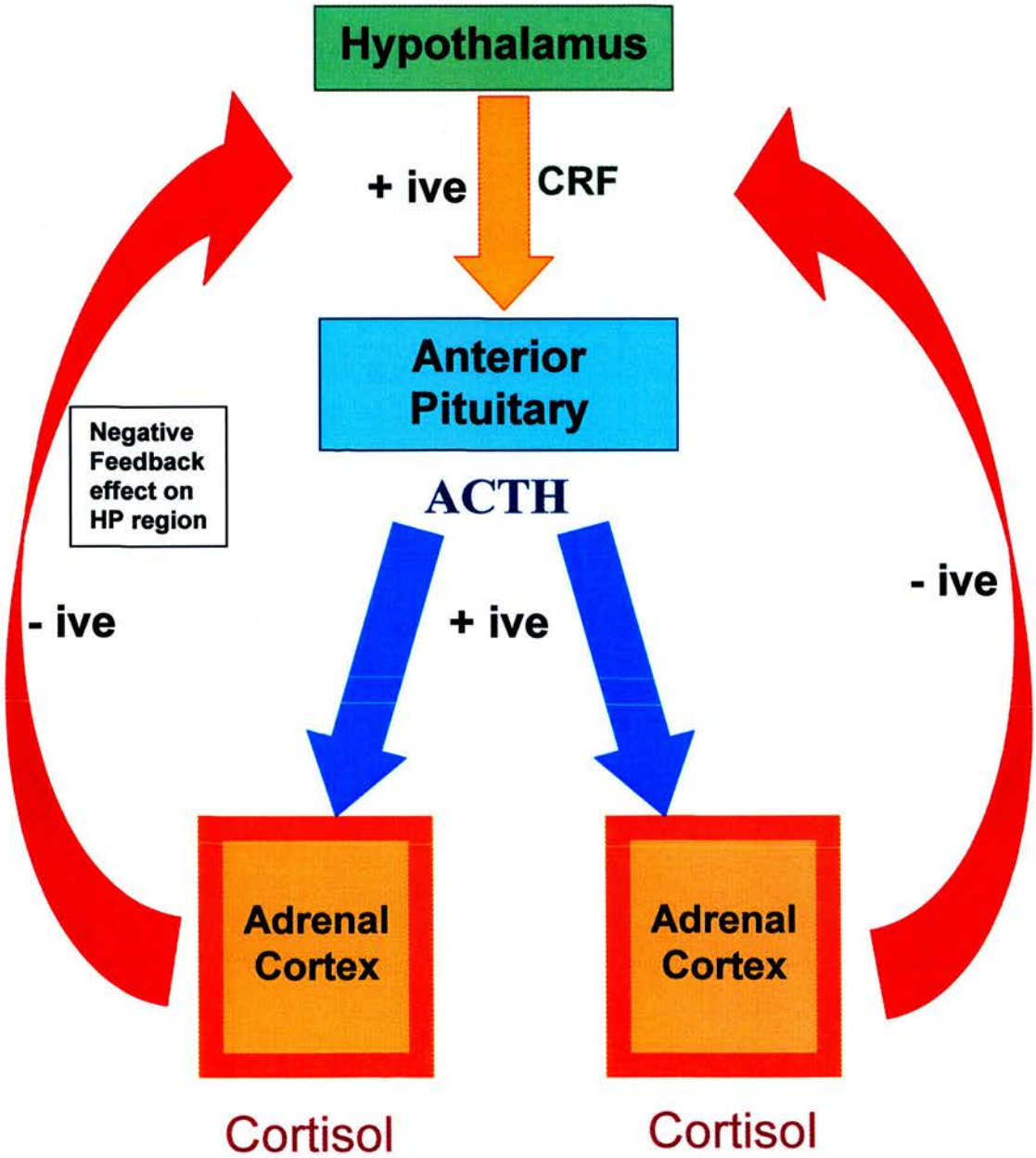

Figure 1.5. Diagrammatic representation of the Hypothalamo-Pituitary-Adrenal Axis.

2.2 The Adrenal Cortex: Structure and Function

Adrenal gland anatomy was first described in 1563 by Bartholomeo Eustachius, but it was not until 1849 that Thomas Addison presented a paper providing evidence of a physiological role (Addison 1849; Eustachius 1574). In 1937, cortisone was identified, and eventually synthesised in 1949.

The adrenal glands are paired pyramidal shaped structures lying above or posteromedial to and occasionally attached to the upper pole of the kidney. Each gland weighs approximately four grams, regardless of age, body weight, or sex. The adrenal glands consist of two functionally distinct units; the adrenal cortex and the adrenal medulla. The adrenal medulla is concerned with the release of epinephrine and norepinephrine and will not be discussed further. The adrenal cortex is that part of the gland responsible for glucocorticoid, mineralocorticoid and androgen production.

2.2.1 Normal Adrenal Physiology

The primary role of the adrenal cortex is the synthesis and release of steroid hormones into the general circulation. The glucocorticoids have numerous effects, exerting their effects throughout the body. Indeed, life cannot exist without their presence in the body.

Glucocorticoids enter the target cells by simple diffusion, and then bind to a specific glucocorticoid receptor which travel from cytoplasm to nucleus, through which their effects are transmitted (Okret et al 1984).

The hormone-receptor complex then binds to deoxyribonucleic acid (DNA), thus regulating gene expression, resulting in either transcriptional activation or inhibition (Rousseau et al 1973). The complex interacts with specific DNA sequences (known

as glucocorticoid receptor elements) (Beato 1989). In this way, glucocorticoids exert their effects on numerous systems in the body.

One of the main effects of glucocorticoids is on carbohydrate metabolism. They enhance glycogen metabolism by activating glycogen synthase (Hornbrook et al 1966) and inhibiting glycogen phosphorylase (Stalmans & Laloux 1979).

Glucocorticoids also have a major effect on glucose metabolism by activating glucose-6-phosphatase and phosphoenolpyruvate carboxykinase, increasing hepatic gluconeogenesis (Exton 1979). Peripheral glucose utilisation is inhibited by glucocorticoids, in part by directly inhibiting glucose transport into cells (Livingston & Lockwood 1975).

Glucocorticoids also activate lipolysis in fat tissues. In chronic glucocorticoid excess, there is a redistribution of body fat, with marked central fat deposition. This lipogenic effect may be due to hyperinsulinaemia resulting from excess glucocorticoids.

Glucocorticoids have an effect on the immune system and can affect inflammatory processes. In supraphysiological doses, glucocorticoids suppress immune responses (Graham & Tucker 1984). However, anti-inflammatory effects are only seen when pharmacological amounts are given (Fauci 1979).

Other effects of glucocorticoids include their influence on muscle and bone metabolism and their effects on mineralocorticoid activity, mood and behaviour, and linear growth and cell development.

2.2.2 Control of Androgen Release by the Adrenal Cortex

The adrenal cortex secretes androgenic steroids, principally dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulphate (DHEAS) and androstenedione (Longcope 1986). These are converted peripherally to testosterone and dihydrotestosterone.

Control of adrenal androgen release remains unclear. Several factors have been implicated. ACTH probably plays an important role since androgen concentrations appear to follow cortisol concentrations, exhibiting the same circadian pattern of secretion (Rosenfeld et al 1975).

Other factors may also be involved, such as an additional pituitary factor. Evidence for this is given by Albertson et al who found that giving ACTH replacement in hypophysectomised chimpanzees did not normalise androgen secretion (Albertson et al 1984). Cunningham et al in 1994 also found that there was dissociation of adrenal androgen and cortisol secretion in Cushing's syndrome (Cunningham & McKenna 1994). Isolates from pituitary extracts have been proposed as possible factors, but this remains controversial.

Other hormones such as prolactin and Insulin-like growth factor 1 (IGF-1) have also been suggested to have adrenal androgen stimulatory roles, but this seems to occur only in pathological states (Higuchi et al 1984).

2.2.3 The Adrenocortical Stress Response

Almost any form of stress, either physical or psychological, results in the release of ACTH followed by an adrenocortical response to this within minutes. The nature of the stress can be anything from acute anxiety to major trauma. The role of this cortisol surge following stress is still not fully understood, but it is known that the release of cortisol rapidly mobilises amino acids and fats, making these available as an energy source and also for the synthesis of glucose for energy utilisation.

2.2.4 Steroid Biosynthesis in the Adrenal Gland

Adrenal steroid hormones are derived from cholesterol, which is converted to pregnenolone by removal of the C-20 side chain by P-450_{scc}. In the cortisol biosynthetic pathway, pregnenolone is then converted to progesterone by

dehydrogenation of the 3-hydroxyl group and isomerisation of the double bond at C-5. This is achieved with the enzyme 3β-hydroxysteroid dehydrogenase.

Cytochrome P-450$_{C17}$ converts progesterone to 17-OHP by hydroxylation of C-17 and also causes cleavage of the residual two-carbon side chain at C-17 on pregnenolone. This allows 17-hydroxylated substrates with their side chain intact to be synthesised as glucocorticoids, and the C19-steroids are directed towards androgen and oestrogen synthesis. Cortisol synthesis is then completed by conversion of 17-progesterone to 11-deoxycortisol by 21-hydroxylation by cytochrome P-450$_{C21}$ enzyme, and finally conversion to cortisol by P-450$_{C11}$.

Adrenal androgens are synthesised from C-17 steroids. Dehydroepiandrosterone is formed from 17-hydroxypregnenolone by the action of P-450$_{C17}$. Androstenedione is formed by side-chain cleavage of 17-hydroxyprogesterone by P-450$_{C17}$.

Mineralocorticoids are synthesised from progesterone by hydroxylation at C-21 by P-450$_{C21}$ to form deoxycorticosterone. This is then converted to corticosterone by hydroxylation at C-11, and finally to aldosterone by methyl oxidation at C-18. These two final steps are catalysed by cytochrome P-450$_{C11}$.

The steroid biosynthetic pathways are summarised in Figure 2.1.

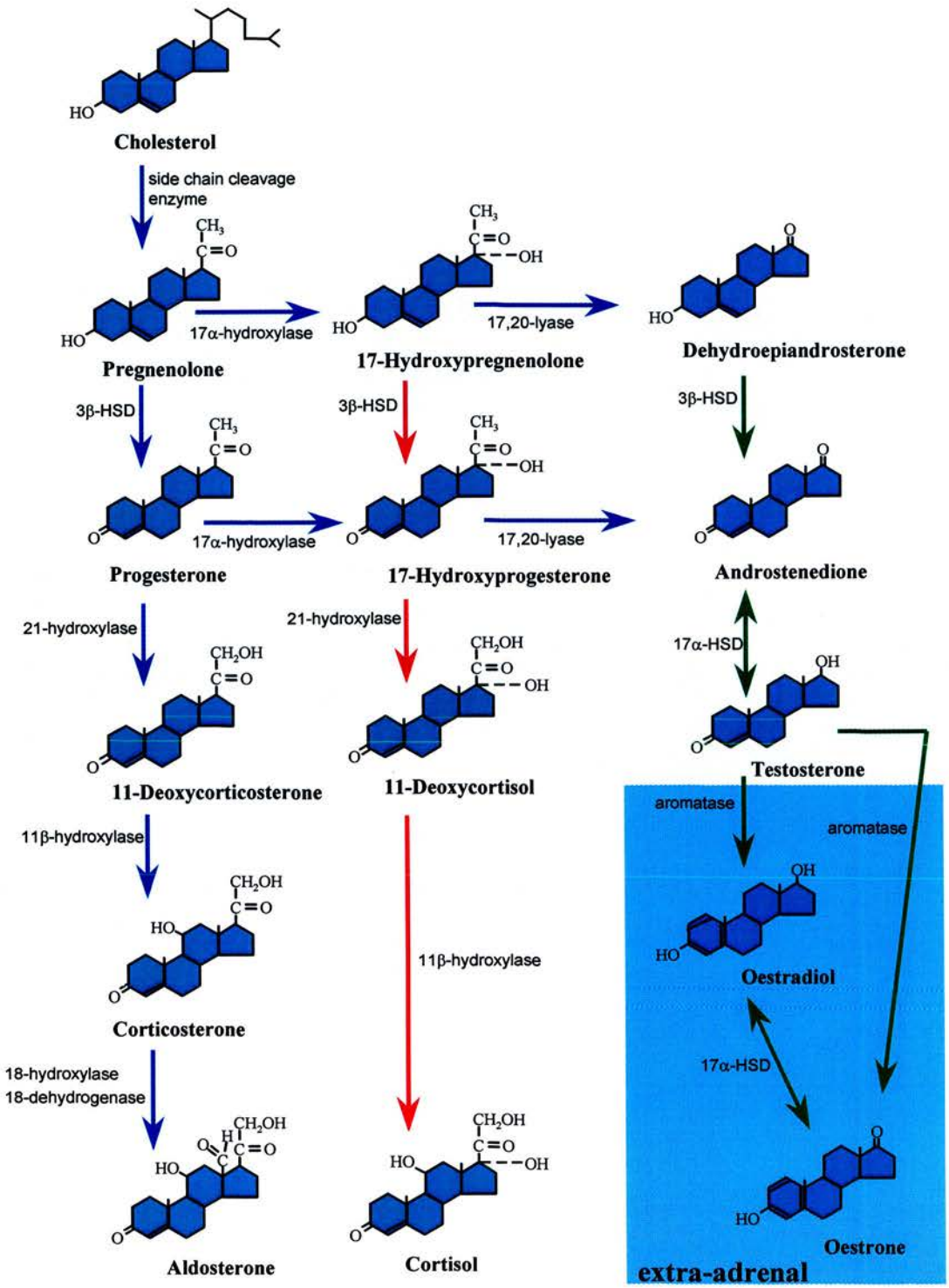

Figure 2.1 Steroid biosynthetic pathway

2.3 Adrenocortical Insufficiency

Adrenocortical deficiency states can originate either from diseases of the adrenal gland itself (primary adrenal insufficiency), or from disorders of the hypothalamo-pituitary region (secondary and tertiary adrenocortical insufficiency). (See Table 2.1). The resultant effect is inadequate adrenocortical function, with inappropriate concentrations of glucocorticoids.

Primary		Secondary
Autoimmune adrenalitis		Post-glucocorticoid therapy
Adrenalectomy		Hypophysectomy
Amyloidosis		Pituitary aplasia
Granulomatous disease:	Tuberculosis	ACTH deficiency
	Histoplasmosis	CRH deficiency
	Sarcoidosis	Idiopathic hypopituitarism
Haemochromatosis		Craniopharyngioma
Adrenoleukodystrophy		Trauma
Haemorrhage		Post cranial irradiation
Neoplastic infiltration		Sarcoidosis
Xanthomatosis		Starvation
Wolman disease (lysosomal acid lyase deficiency)		Anorexia nervosa
Congenital adrenal hyperplasia		
Congenital adrenal hypoplasia		
ACTH receptor defect		

Table 2.1. Causes of adrenocortical insufficiency

2.3.1 Primary Adrenocortical Insufficiency

In primary adrenocortical failure (resulting from primary adrenocortical damage), a lack of cortisol feedback results in elevated levels of ACTH; a phenomenon used to advantage in diagnosis. Mineralocorticoid secretion is also affected, resulting in salt wasting, with hyponatraemia and raised potassium concentrations as well as hypoglycaemia. The clinical features of adrenocortical insufficiency are dependant on the rate of loss of adrenal function. In general, the history of primary adrenocortical insufficiency is short, and the features are severe and acute, the patient presenting in adrenal crisis. The patient is acutely unwell, hypotensive and volume depleted, with characteristic changes in the electrolyte levels.

2.3.2 Secondary Adrenocortical Insufficiency

In contrast to primary adrenocortical insufficiency, the symptoms of secondary or tertiary adrenocortical insufficiency are usually insidious and non-specific, and may exist for many years before the patient develops acute adrenal failure. Salt wasting does not occur. Tertiary adrenocortical insufficiency results from hypothalamic damage, whereas SAI can result from any process affecting the pituitary gland and affecting ACTH secretion (Table 2.1). In practice, it is difficult to differentiate between secondary and tertiary insufficiency.

2.3.3 Diagnosis of Adrenocortical Insufficiency

The features of Addison's disease tend to be obvious at presentation, with the characteristic features of hyperpigmentation and reverse pigmentation, biochemical derangement (hyponatraemia and hyperkalaemia), a poor cortisol response to synacthen, and raised circulating concentrations of both ACTH and plasma renin. By contrast, the symptoms of SAI, which include tiredness, lethargy, malaise, and increased frequency of trivial upper respiratory infections of prolonged duration, are

insidious and very similar to those which can occur after cranial irradiation and radiotherapy.

A specific biochemical test does not exist for SAI (see below) and diagnosis is difficult, unless the patient has florid symptoms.

2.4 Adrenal function tests in hypoadrenalism

In order to determine the integrity of the HPA axis with reference to diagnosing cortisol deficiency states, steroid hormones are either measured at various times of the day, or provocation tests are performed which stress the axis and give an estimate of the HPA reserve. In general, little further information can be gained in diagnosing secondary hypoadrenalism by measuring ACTH. At the time of writing, there are no assays available to sensitively determine CRH concentrations. However, the usefulness of measuring circulating CRH is questionable, as concentrations in peripheral blood are low.

Physiological tests	Provocation tests
random plasma/salivary cortisol/adrenal steroids	cortisol response during insulin hypoglycaemia
diurnal variation in plasma/salivary cortisol	cortisol response during ACTH stimulation
plasma/salivary cortisol/steroid profiles	adrenal steroid response to metyrapone
urinary steroid profiles	Cortisol and ACTH responses to Corticotrophin releasing hormone

Table 2.2. Summary of tests used for detecting adrenocortical Insufficiency

2.4.1 Physiological tests used to assess Adrenocortical Function

These tests assess the HPA axis by measuring steroid hormones produced from the adrenal cortex without subjecting the patient to any stimulus that may influence steroid production. In practice this is very difficult to perform, as even simple stresses (such as the thought of venepuncture) have been shown to affect steroid secretion.

Several attempts have been made to establish the usefulness of physiological tests in assessing adrenal function (Allin et al 1984; Hagg et al 1987).
Early morning serum cortisol concentrations, taken between 7a.m and 9a.m may be helpful in diagnosing adrenocortical insufficiency (Hagg, Asplund, & Lithner 1987). However, this method can be unreliable, as in many cases, the actual time of peak for each individual may be missed.
The use of venepuncture to assess adrenal function can also provide erroneous results, particularly in children, as the stress of the procedure, or even the anticipation may affect adrenal cortisol release.

Urinary steroid profiles and urinary free cortisol determinations have been advocated as useful non-invasive methods of assessing adrenal function in children, provided that the technique of urine collection is meticulous (Gomez et al 1991; Honour et al 1991). However, in younger children this may not be possible, as collection of urine is difficult to perform accurately.

2.4.2 Provocation tests used in the diagnosis of Adrenocortical Insufficiency

Provocation tests assess maximal steroid release to a specific stimulus, such as hypoglycaemia, metyrapone inhibition, or ACTH stimulation. The tests are

essentially non-physiological and assess adrenal reserve rather than actual function.

Since its introduction (Jacobs & Nabarro 1969), the insulin induced hypoglycaemia test (ITT) has been looked upon as the "gold standard" in many studies evaluating HPA function. However, there have been reports of patients who clinically were felt to be cortisol deficient, but who had a normal cortisol response to hypoglycaemia and an abnormal response to synacthen (Borst et al 1982). Perhaps more importantly, the ITT is not without risk, particularly in patients with adrenocortical insufficiency.

The ACTH stimulation test is frequently used to assess adrenocortical function (Lashansky et al 1991; Lindholm & Kehlet 1987). The standard cortisol response to a pharmacological dose (250μg) of synthetic ACTH analogue as Synacthen, 1-24 ACTH (Ciba-Geigy) is determined. The ACTH dose is several hundred times greater than the concentrations reached under physiological conditions. The rationale for using ACTH stimulation in the assessment of SAI is that an already under stimulated adrenal gland will show a sluggish response to exogenous ACTH. In general, the cortisol response to ITT and Synacthen are broadly comparable (Jackson et al 1994). However, discrepancies have been noted, making interpretation difficult in individual patients. Moreover, some patients with SAI have been shown to have a normal response to pharmacological doses of ACTH analogue (Cunningham, Moore, & McKenna 1983).

Alternatively, the cortisol response to infusions of supposedly physiological amounts of ACTH may be assessed (Landon et al 1964; Landon 1967). More recently, in 1991, Crowley et al used a low dose ACTH test to assess adrenocortical function (Crowley et al 1991; Dickstein et al 1991). Low doses of intravenous Synacthen (500ng/1.73m^2 body surface area) were shown to produce similar results when

compared with the standard 250μg ACTH test in normal subjects. Using a lower, more physiological dose may provide more accurate information of adrenal function.

The metyrapone test has been used to specifically assess adrenocortical reserve and diagnose ACTH deficiency (Best et al 1980; Dickstein et al 1986; Gans & Ulstrom 1962; Limal et al 1976; Strott et al 1969). Metyrapone is a specific 11β-hydroxylase inhibitor (See Figure 2.1). Administration of metyrapone results in a fall in plasma cortisol concentration that increases hypothalamo-pituitary drive, causing increased production of steroid hormones proximal to the block. These can be measured in plasma (11-deoxycorticosterone and 11-deoxycortsol) and, more conveniently in children, in urine (Ochs & Mulhern 1988). Metyrapone is not without potentially serious side effects, as it effectively renders patients hypoadrenal. The original metyrapone test, described in 1959, was very unpleasant, and potentially dangerous. However, a single oral dose test has been used in children producing minimal adverse effects (Jubiz et al 1970).

Finally, the HPA axis may be assessed using the cortisol response to CRH (Dahl et al 1992; Ross et al 1986). The effectiveness of this method is currently under evaluation, but apart from its usefulness in differentiating hypothalamic from pituitary lesions, it is likely to have similar problems as the ACTH test.

2.5 Radioimmunoassay of Steroid Hormones

Radioimmunoassay (RIA) was first described in 1960 following the development of techniques that rendered the handling of radioisotopes relatively safe (Berson & Yalow 1956). Isotope usage enabled the detection of very small quantities of substances. Since hormones are present in the body in very small amounts, this development revolutionised endocrinology. The use of antibody as a binder was developed in 1960 by Yalow and Berson when RIA for insulin was developed (Yalow & Berson 1960). Subsequently, Ekins developed a competitive protein-binding assay for thyroxine (Ekins 1960). These two immunoassays formed the basis of further immunoassay development.

The basic concept of RIA is one of competitive binding of antibody to antigen or ligand. The antibody binds to antigen in a reversible reaction. If more than one type of ligand is present, there is competitive binding between the two ligands and the antibody binding sites. The distribution of antigen between the bound and free phase depends on the total amount of antigen present. In this way, the total amount of antigen present can be calculated.

The principles of RIA are basically the same in all assays. A standard curve is constructed by incubating fixed amounts of antibody and labelled antigen with varying concentrations of unlabelled ligand. From this, a curve is plotted of percentage of antibody bound against concentration of unlabelled ligand. If plotted on a log scale, a sigmoid curve is obtained.

The accepted requirements for a RIA are the use of a purified antigen, a purified tracer ligand, specific antibody, and a robust method for distinguishing between or separating bound and free ligand.

It is essential that a highly purified ligand is used in order to measure the antibody bound and free fractions. Radio-iodine isotope (^{125}I), is highly specific, is easier to detect and is cheaper to detect than the alternative tritium. Expensive scintillation fluid is not needed with iodine label. Instead multihead counters are used which allow for rapid sampling.

Improving sensitivity of an assay may be achieved by altering the concentration of antibody, the total volume of antibody and ligand, and the environment in which the assay is performed (i.e. the temperature). The specificity of an assay can be improved by changing the characteristics of the antibody such as improving binding characteristics and reducing cross-reactivity.

2.6 The Use of Salivary and Capillary Steroids

2.6.1 Salivary Steroids

Saliva as a medium is attracting interest as an investigative and screening tool. It is increasingly being used to monitor antibody levels to both viruses and antibodies, concentrations of drugs (e.g. phenytoin), and to measure steroid hormones such as cortisol, 17-OHP, androstenedione, testosterone, and DHEA (Malamud D. 1992).

The first assays of steroids in saliva were performed in 1959, when Shannon et al performed pioneering work in measuring 17-hydroxycorticosteroids in the parotid fluid of healthy adult males using a calorimetric method (Shannon et al 1959). It was shown then that there was a diurnal variation of the 17-hydroxycorticosteroids, and also that there was a response of salivary steroids to corticotrophin. However, large volumes of saliva were required (over 10 ml) because of assay insensitivity, and a collecting device was necessary; possibly because of this, the challenge to use saliva in endocrinology was not accepted!

With the advent of RIA, hormone assays have become much more sensitive, resulting in smaller sample requirements (less than 200 μl). Much of this later work on the use of saliva was performed at the Tenovus Institute for Cancer Research in Cardiff (Riad-Fahmy et al 1982). Several other workers have now developed salivary steroid assays for use in particular aspects of clinical and psychological medicine (Al-Ansari et al 1982; Bailey & Heitkemper 1991; Dabbs et al 1991; Hiramatsu 1981; Kirschbaum & Hellhammer 1989), or have adapted serum assays for use in saliva (Al-Ansari, Perry, Smith, & Landon 1982).

The use of saliva has advantages over serum or plasma and even urine (see Table 2.3). Collecting saliva is relatively simple and stress free. Initial collecting devices were unsatisfactory due to the steroids adhering to the porous material used (Walker et al 1990). However, use of these collecting devices ("salivettes") can be helpful in situations requiring long-term storage of samples at room temperature (Chen et al 1992). The simplest method of collection has been either by aspirating saliva from the oral cavity following citric acid stimulation, or by simply spitting directly into clear sterile containers.

Saliva collection does not require medical or nursing staff supervision, allowing it to be performed in the stress-free environment of the patients' own home. This is particularly attractive in the paediatric population, since it avoids the problems associated with venepuncture, and its attendant ethical and practical constraints, such as multiple sampling over long periods.

Uncertainty still exists regarding the validity of the methods employed in assaying hormones in saliva. There does still seem to be an emotive bias against the use of saliva possibly due to the clinicians' perception that a blood sample is somehow "better". Saliva is often compared with sputum, which also tends to avert clinicians from collecting it.

Free plasma hormone concentrations are thought to reflect the biologically active fraction of the hormone but are difficult to measure; the procedures involve ultrafiltration or equilibrium dialysis, both of which are technically demanding and time consuming. Measurement of hormones in saliva relates to the free, unbound circulating level of the hormone rather than total levels. Poteczin et al have demonstrated excellent correlation between plasma free progesterone and salivary progesterone concentrations during pregnancy and Smith et al have shown a close relationship between free testosterone measured in plasma and testosterone in saliva (Smith et al 1979). Salivary cortisol concentrations have been shown to be directly proportional to serum free cortisol concentrations (Reid et al 1992; Riad-Fahmy, Read, Walker, & Griffiths 1982; Tunn et al 1992; Umeda et al 1981; Vining et al 1983a; Vining & Mcginley 1982; Walker et al 1976; Woodside, Winter, & Fisman 1991).

Advantages	Disadvantages
Simple to perform sampling	Unfavoured by clinicians
Stress free	Requires sensitive assays
Useful in multiple sampling	Concerns that salivary flow rates may affect levels
Particularly useful in children	May not reflect true free cortisol levels
Simple to assay, as binding proteins are not present	

Table 2.3. Advantages and Disadvantages of the Use of Saliva in Clinical Practice

One argument against the use of saliva is the claim that salivary hormone concentrations are inversely related to salivary flow rate. There is now good experimental evidence that this is not the case (Riad-Fahmy, Read, Walker, & Griffiths 1982).

A recognised problem is falsely high salivary steroid concentrations due to blood contamination. Care is required during saliva collection, samples should not be collected after tooth-brushing, which may damage the buccal mucosa and gum margin, resulting in blood contamination (Schramm et al 1993).

There have to date been many assays either developed or adapted for measuring cortisol in saliva (Hiramatsu 1981). These assays are simple, sensitive, and specific. To date, however, the majority of these assays have been developed using 'in-house' reagents for research purposes (Landon et al 1982), although some commercial serum assays have been adapted for use with saliva (Al-Ansari, Perry, Smith, & Landon 1982).

2.6.1.1 Application of Salivary Steroid Assays

Salivary cortisol concentrations have been shown to follow the same diurnal pattern as do serum concentrations (Bailey & Heitkemper 1991; Walker et al 1982). Salivary cortisol has also been shown to rise in response to Synacthen and insulin induced hypoglycaemia with a similar incremental rise compared with serum values (Laudat et al 1988).

Salivary cortisol concentration correlates extremely well with plasma free cortisol concentration, suggesting that they are as valuable as plasma concentrations (Reid, Intrieri, Susman, & Beard 1992; Tunn, Mollmann, Barth, Derendorf, & Krieg 1992; Umeda, Hiramatsu, Iwaoka, Shimada, Miura, & Sato 1981; Vining et al 1983b;

Vining & Mcginley 1982; Walker, Riad-Fahmy, & Read 1976; Woodside, Winter, & Fisman 1991).

Moreover, salivary cortisol correlate extremely well with serum total cortisol and free cortisol (Riad-Fahmy, Read, Walker, & Griffiths 1982; Tunn, Mollmann, Barth, Derendorf, & Krieg 1992). Therefore, salivary cortisol concentrations accurately reflect serum free and total cortisol concentrations.

Measurement of salivary cortisol in response to ACTH provides a useful assessment of adrenal reserve (Peters et al 1982b). In fact, the percentage rise of cortisol in saliva is greater than in serum (Riad-Fahmy, Read, Walker, & Griffiths 1982).

One important concern with regard to salivary cortisol is the conversion of cortisol to cortisone in the submaxillary gland in the mouth due to the presence of 11β-hydroxysteroid dehydrogenase. This may in theory affect the relationship between plasma free and salivary cortisol. Studies performed suggest that there is excellent correlation between plasma free and salivary cortisol (Brooks & Brooks 1982; Peters, Walker, Riad-Fahmy, & Hall 1982b; Peters et al 1982a; Umeda, Hiramatsu, Iwaoka, Shimada, Miura, & Sato 1981). These studies imply that the correlation holds true despite cortisol/cortisone interconversion in saliva.

When studying different age groups and sexes, it has been shown that the reference ranges for early morning samples in males, females and children are not significantly different. Moreover, the range of values in different studies in which different assay techniques were used, were generally in good agreement.

Sex hormone binding globulin (SHBG) has been detected in very small amounts in saliva. However, the effect on cortisol levels in saliva is minimal (Selby et al 1988). SHBG levels may also be higher in blood contamination; this may be another factor resulting in falsely high salivary cortisol levels.

Steroids such as 17-OHP and androstenedione (Turkes & Read 1982) have also been measured in saliva, but unfortunately there is little normative data available for these steroids (Turkes & Read 1982; Young et al 1988b). Some work has been done on the use of salivary 17-OHP in monitoring children with congenital adrenal hyperplasia (CAH) (Young et al 1988a).

Other steroid hormones such as progesterone and oestrogens have also been measured in saliva. These have been used in monitoring fertility, and are a useful adjunct to fertility control.

2.6.2 Capillary Steroids

The use of capillary blood spot samples is becoming more widely used as a method of patient monitoring. The use of home blood glucose monitoring in diabetic patients is a major part of diabetic monitoring and blood glucose control.

Blood spot 17-OHP profiles have been used for some time to monitor the control of patients with congenital adrenal hyperplasia (Young, Robinson, Read, Riad-Fahmy, & Hughes 1988a). The use of androstenedione in monitoring CAH has also been reported, and the use of androstenedione profiles in conjunction with 17-OHP has been advocated as a more accurate method of assessing management of these patients (Hughes & Winter 1978).

Measurement of blood spot cortisol in the screening of children at risk of developing SAI therefore seems attractive, in a similar way to saliva. However, blood spot sampling is more invasive than saliva collection. There have been no reports on the use of blood spot cortisol estimations to date.

2.7 Adrenal Function in Childhood Cancer Survivors

There have been surprisingly few reports of HPA dysfunction in childhood cancer survivors. In leukaemia survivors who have not suffered relapse, there are, as far as we are aware, no reports of SAI. There is no evidence of significant disruption of spontaneous ACTH or cortisol secretion in children who had received low dose cranial irradiation (18-24 Gy) (Crowne et al 1993; Wallace et al 1990) when compared with normal children (Wallace et al 1991).

In BMT survivors, the incidence of HPA dysfunction was reported to be 24% (Sanders, Pritchard, Mahoney, Amos, Buckner, Witherspoon, Deeg, Doney, Sullivan, Appelbaum, Storb, & Thomas 1986). However, this was based on the metyrapone test, measuring 11-deoxycortisol levels post-metyrapone. None of the patients in this study were symptomatic.

In brain tumour survivors, the expected incidence of HPA dysfunction should be significantly higher. However, once again there are few reports on HPA abnormalities. In 1989, a report suggested a prevalence of one third of adult patients with multiple endocrine deficits having ACTH deficiency (Littley, Shalet, Beardwell, Ahmed, Applegate, & Sutton 1989). In childhood brain tumour survivors, there are reports in which children are reported to require steroid replacement therapy, with a presumed diagnosis of HPA dysfunction (Brown, Lee, Eden, Bullimore, & Savage 1983).

The prevalence of ACTH deficiency in adult cancer survivors is said to be similar to that of gonadotrophin deficiency (Littley, Shalet, Beardwell, Ahmed, Applegate, & Sutton 1989). However, this does not seem to be the case in children (personal observation), although this has not been conclusively shown. Recognising ACTH deficiency is difficult as the clinical features tend to be non-specific, and very similar

to the symptoms experienced post-radiotherapy. This can lead to a delay in diagnosis. Moreover, the diagnosis of ACTH deficiency may not always be possible using conventional dynamic tests (Tsatsoulis et al 1988).

In 1968 a group looked specifically at pituitary-adrenal function in patients with untreated pituitary tumours and found that the response to various stimuli (metyrapone, corticotrophin, pyrogen, insulin and surgical stress) varied (Jenkins & Else 1968). However, all had an adequate response to surgical stress. They concluded that the responses may not give an accurate indication of impairment of hypothalamo-pituitary-adrenal axis, and may not indicate whether the patient will have an appropriate physiological stress response.

2.7.1 Problems in Clinical and Laboratory Diagnosis

The main problems in diagnosis of SAI in cancer survivors are the lack of clinical pointers to diagnosis, coupled with interpretative difficulties of the commonly used diagnostic tests. This makes definitive diagnosis difficult and leads to either unacceptable false positive or false negative diagnosis of SAI, hence the need for the study.

2.7.2 Design of Study

The aim of the study was to develop a screening technique for detection of hypoadrenalism in children at risk of developing SAI. The study consisted of three phases:

1. The development of a relatively non-invasive test for assessing adrenocortical function using saliva and/or capillary blood samples.

   For this, a sensitive radioimmunoassay for measuring cortisol was developed. Assays for salivary 17-OHP and androstenedione were adapted from existing capillary blood spot samples.

The measurement of these three steroid hormones may be able to provide more sensitive indicators of developing SAI.

2. To obtain normative data for these three steroid estimations in both saliva and capillary blood spots from 24 hour profiles in children.
3. To assess adrenocortical function in childhood cancer survivors, using currently available diagnostic techniques, as well as using saliva and capillary blood spot profiles.

3 Subjects, Materials and Methods

3.1 Control Population

3.1.1 Recruitment of Control Subjects

Ethical approval was granted to approach schools within the area surrounding the Royal Hospital for Sick Children (RHSC), Glasgow, for volunteers to provide samples of saliva. Permission was then given by the Regional Education Authority to contact schools in this area. Ethical approval was, however, not granted to obtain capillary blood spot samples from healthy volunteers including the siblings of childhood cancer survivors. The Yorkhill ethical committee felt that finger prick sampling on healthy children was too invasive and not in the best interests of the volunteers.

Nine schools were approached, and of these, four schools agreed to allow their children to take part in the study.

3.1.2 Field Work

Four schools agreed to take part; three were primary schools and one a secondary school. The secondary school and one of the primary schools served an area close to the University of Glasgow, and for this reason there was a range of different ethnic groups in those schools, predominantly Middle Eastern and Asian, as well as Caucasian.

Children were excluded from the study if they were known to be unwell, were suffering from a chronic illness, or were on any long-term treatment (e.g. steroid inhalers).

An information sheet and consent form was given to each child in the primary schools and to all children up to the age of 15 years in the secondary school (appendix 1a, b, c, d; 2). In two of the primary schools the head teachers felt that the parents should be offered the opportunity to discuss the project with the author for further information. Consequently, evening meetings were arranged where the interested parents could obtain further information and had the opportunity of asking further questions regarding the study. In total, 340 children gave written consent to participate in the study. Following this, each school was visited by the author and Miss Wendy F. Paterson (WFP, Auxologist in the Department of Child Health, RHSC, Yorkhill, Glasgow). The children were measured by WFP and given collecting bottles and instruction sheets regarding saliva collection and storage.

The children were also asked to provide samples of saliva if they were unwell (e.g. pyrexial with a temperature greater than 39°C, and unable to attend school) at any time over a 2 month period.

3.1.3 Auxology of the Control Population

The control subjects were measured using a portable free-standing stadiometer. All children were stretched in order to reduce the measurement changes due to posture. The children were weighed using portable electronic weighing scales and a portable stadiometer. Height and weight was measured by a single observer (WFP). Pubertal staging was not performed.

3.1.4 Collection of Salivary Samples from the Control Population

Saliva samples were collected at home in control subjects. The children were asked to rinse their mouth with clear water for approximately 30 seconds, and then to empty their mouths. They were then asked to spit directly into clear plastic tubes

after waiting for one minute. Once they had collected between one and two millilitres of saliva, they were asked to freeze the sample.

The subjects were asked to avoid collecting samples for at least one hour after they had brushed their teeth, and after eating or drinking (Appendix 2). Up to six samples of saliva were collected over 24 hours, at the following times: 8 a.m.; 11 a.m.; 5 p.m.; 8 p.m.; 11 p.m. (if the subjects are still awake), and 8 a.m. the following day.

3.2 Patient Population

3.2.1 The West of Scotland Experience

The haematology and oncology unit at the RHSC, Glasgow is a tertiary referral centre, providing services to the West of Scotland, for all childhood malignancies. (Fig. 3.1) The regional bone marrow transplant unit for Scotland is also located in RHSC Glasgow and the oncology centres in Edinburgh and Aberdeen currently refer all their patients to Glasgow for bone marrow transplantation.

3.2.1.1 Leukaemias

Since 1975, 462 children (275 boys, 187 girls) have been treated in Glasgow with leukaemia of various types (Fig. 3.2, 3.3). There has been a steady increase in the number of patients treated since 1975. The commonest haematological malignancy treated in Glasgow is ALL, accounting for nearly 80% of haematological malignancies treated. The survival rate for childhood leukaemias for children treated in Glasgow has continued to improve and is currently 72% in females, and 65% in males treated between 1985 and 1989 (greater than 10 year survival). The overall survival rate for all children treated between 1975 and 1994 is 25% in males and 28% in females (Fig. 3.4, 3.5, 3.6, 3.7). The charts show a steady improvement in survival over time, presumably due to improved therapeutic techniques.

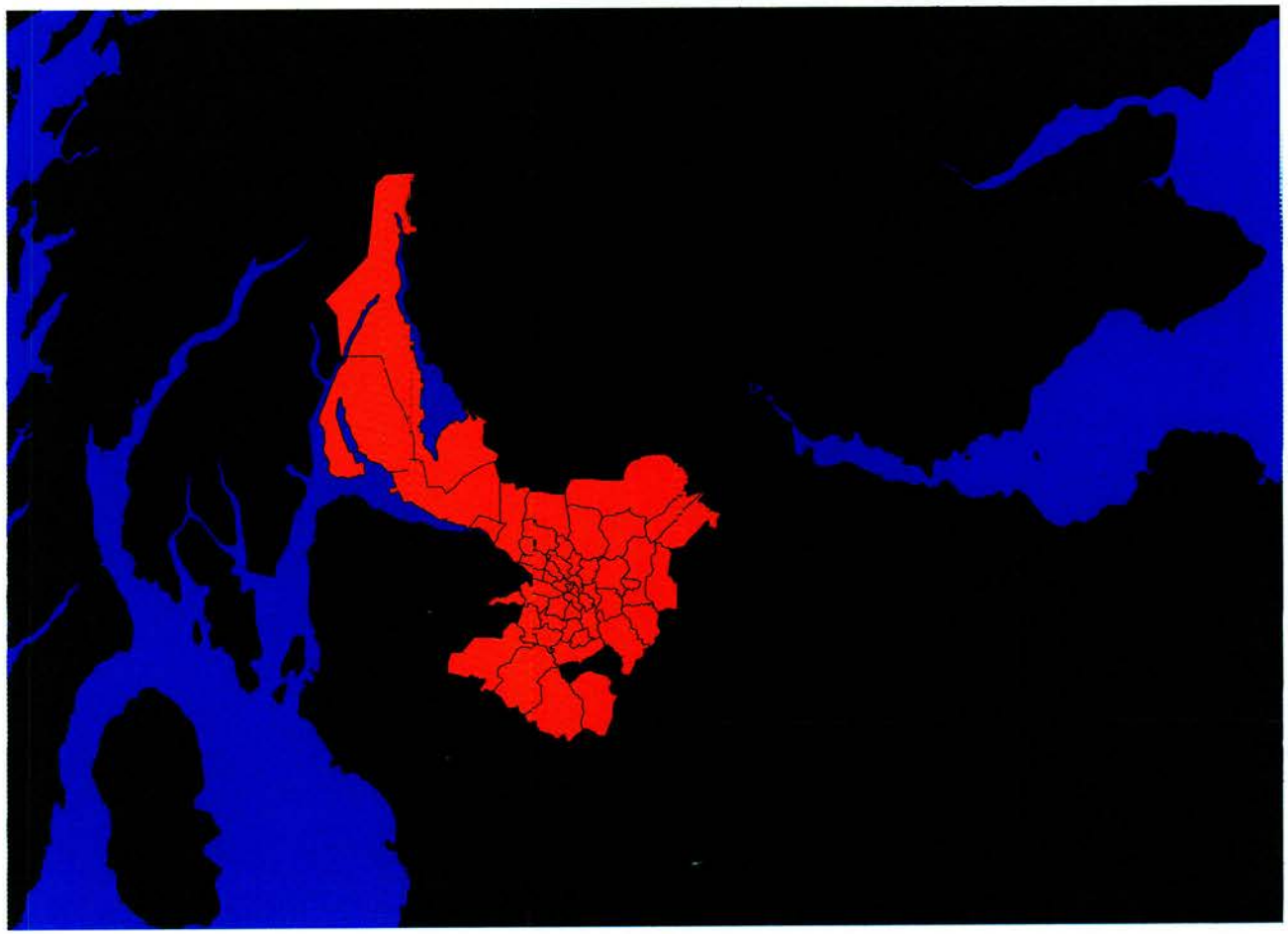

Figure 3.1. Main catchment area served by the Royal Hospital for Sick Children Glasgow (shown in Orange)

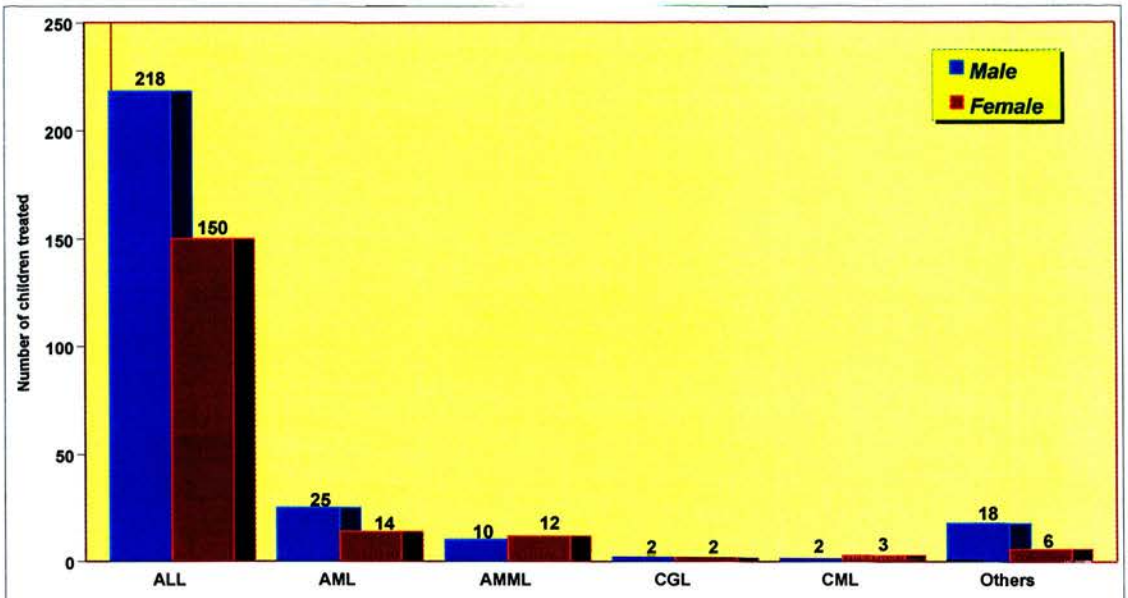

Figure 3.2. Diagnostic Breakdwn of children treated for haematological malignancy in RHSC, Glasgow: 1975 to 1994.

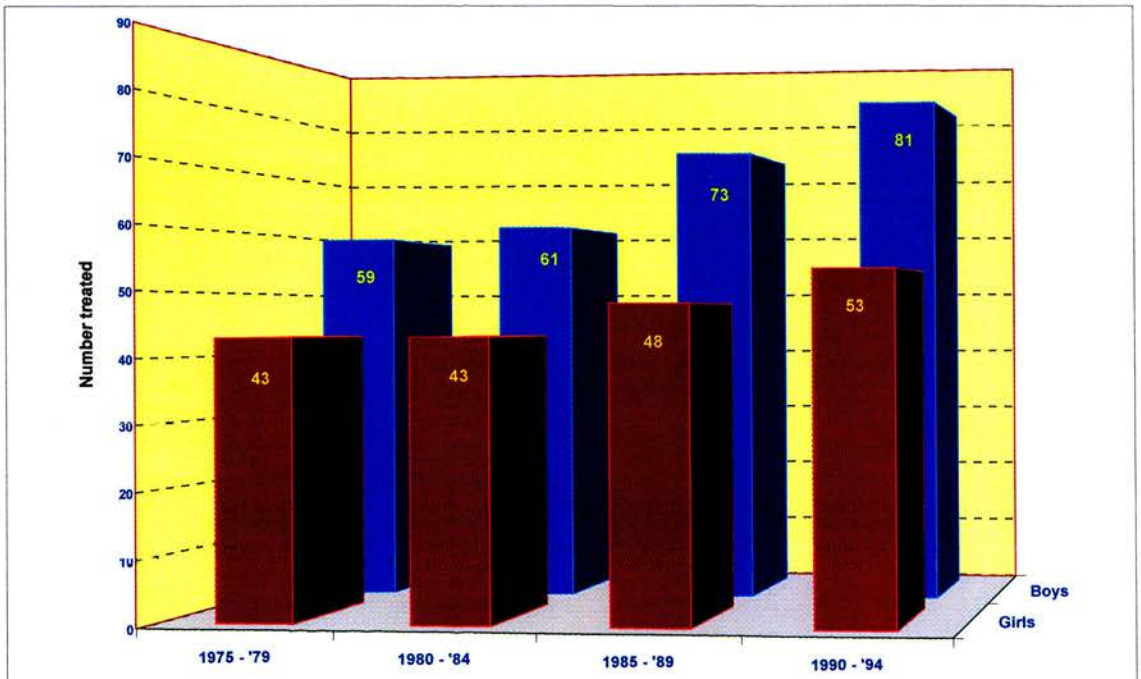

Figure 3.3. Number of Children Treated for Leukaemia - RHSC, Glasgow: 1975 to 1994

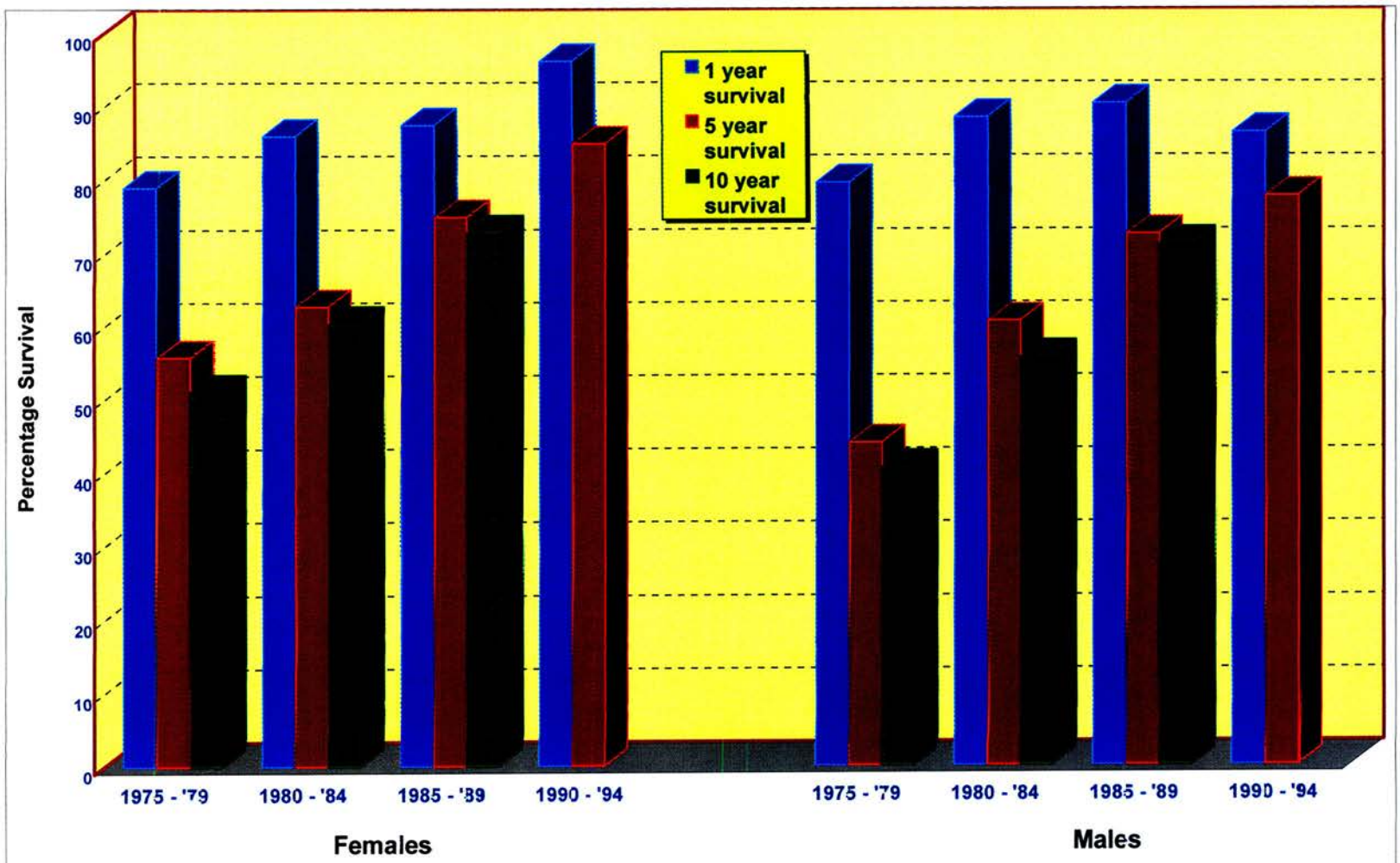

Figure 3.4. Childhood leukaemia survival rates - RHSC, Glasgow: 1975 to 1994

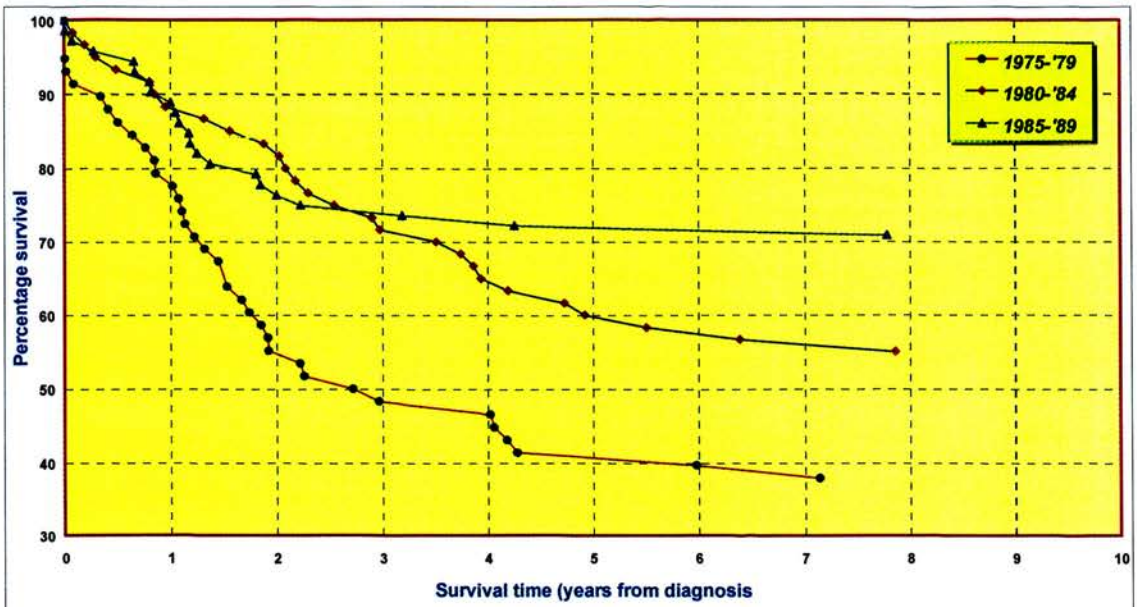

Figure 3.5. Cumulative survival curves for haematological malignancies in males treated in RHSC, Glasgow; 1975 to 1989.

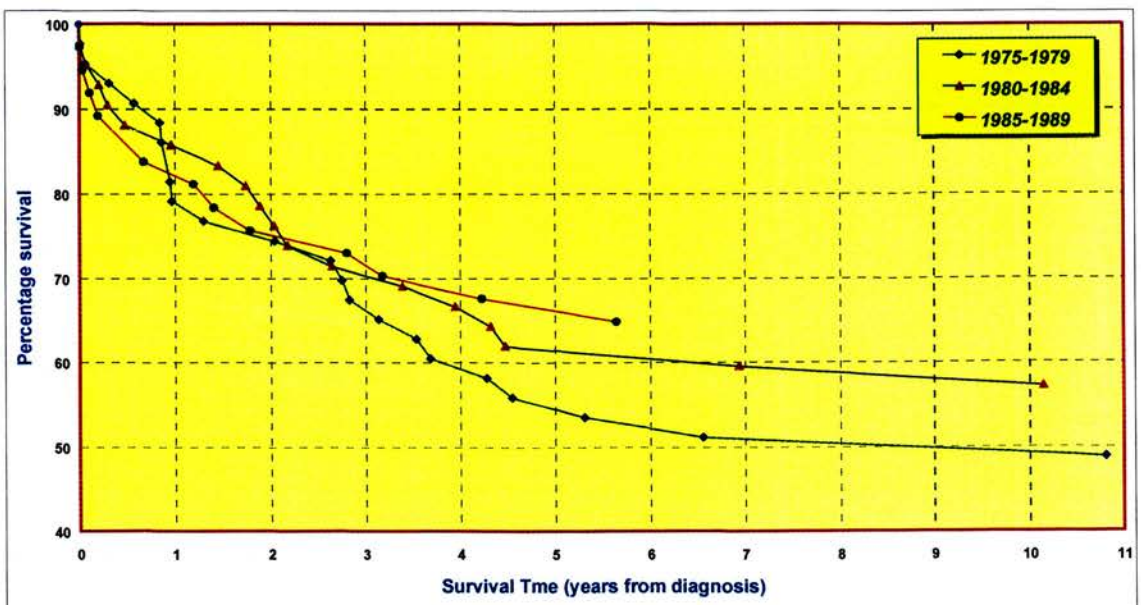

Figure 3.6. Survival curves for haematological malignancies in females treated in RHSC, Glasgow; 1975 to 1989

3.2.1.2 Bone Marrow Transplantation

Bone marrow transplantation as a treatment for childhood leukaemias and certain tumours (e.g. neuroblastomas) was first performed in Glasgow in 1975. Since then, bone marrow transplantation has been performed in 88 children (35 girls, 53 boys). Most transplants have been performed for treatment of relapse of leukaemia (commonly acute lymphoblastic leukaemia) (Fig. 3.7). Other indications include high risk leukaemia (AML, T-cell ALL) in first remission, aplastic anaemia, and selected solid tumours (e.g. neuroblastoma).

The regimen for bone marrow transplantation in malignant disease in Glasgow consists of conditioning with cyclophosphamide and total body irradiation (TBI), giving 1440 cGy in 8 fractions.

The overall 10 year survival rate at RHSC, Glasgow for these children is 57% (Fig. 3.8). However, for the different diagnostic groups treated with BMT, the mortality rate is variable, with the best survival rate for boys with ALL (Fig. 3.9).

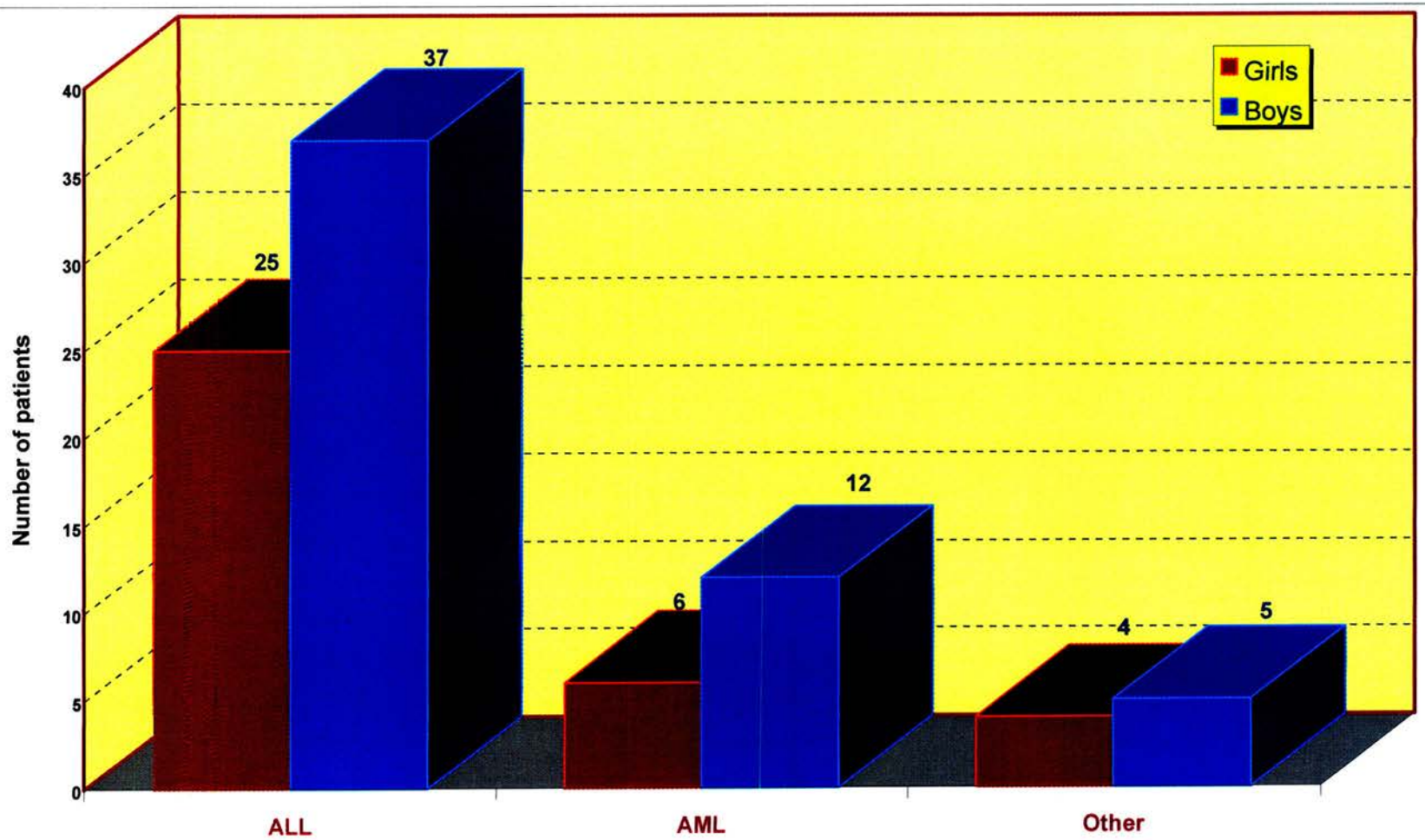

Figure 3.7. Reasons for bone marrow transplantation - RHSC, Glasgow. 1975 to 1994

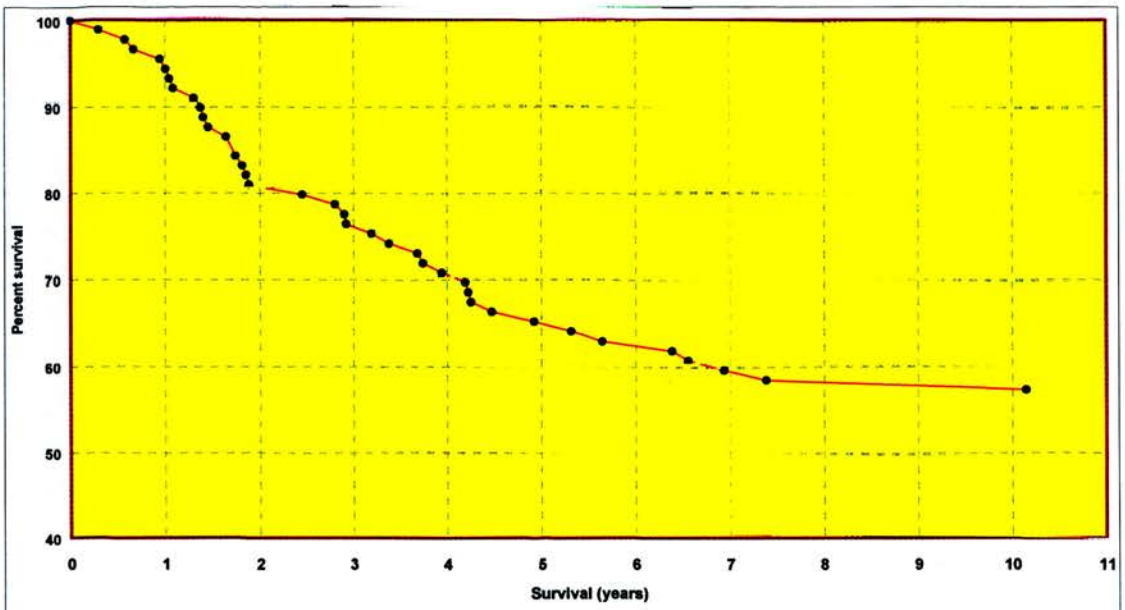

Figure 3.8. Overall survival rates for bone marrow transplant recipients treated in RHSC, Glasgow: 1975 to 1994

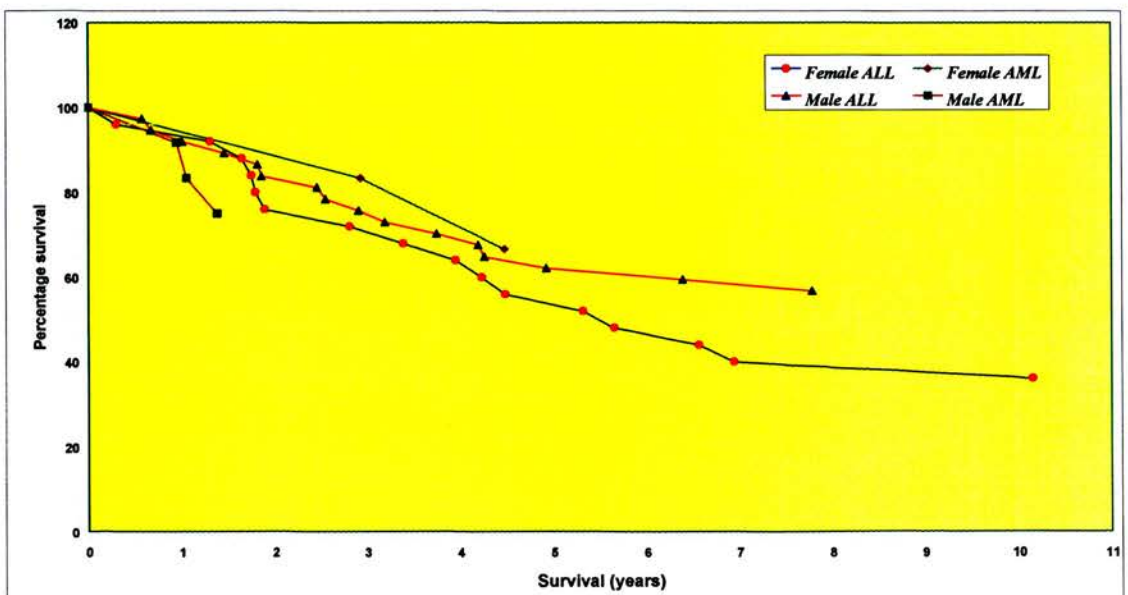

Figure 3.9. Overall survival rate for ALL and AML patients treated with bone marrow transplantation in RHSC, Glasgow: 1975 to 1994

3.2.1.3 Brain Tumours

RHSC Glasgow is a tertiary referral centre for the management of childhood brain tumours of various types (Fig. 3.10; 3.11). Surgical treatment takes place in the nearby Institute of Neurosciences, with subsequent management under the care of the paediatric oncologists and radiotherapists. The commonest brain tumours treated are medulloblastoma and astrocytoma, accounting for 124 out of 212 (58%) children treated. Overall, 105 boys and 107 girls have been treated between 1975 and 1995. The overall survival rate is comparable with national figures; the five year survival rate is approximately 60%. The cumulative survival rates are also comparable with improvements in survival for brain tumours overall (Fig. 3.12, 3.13).

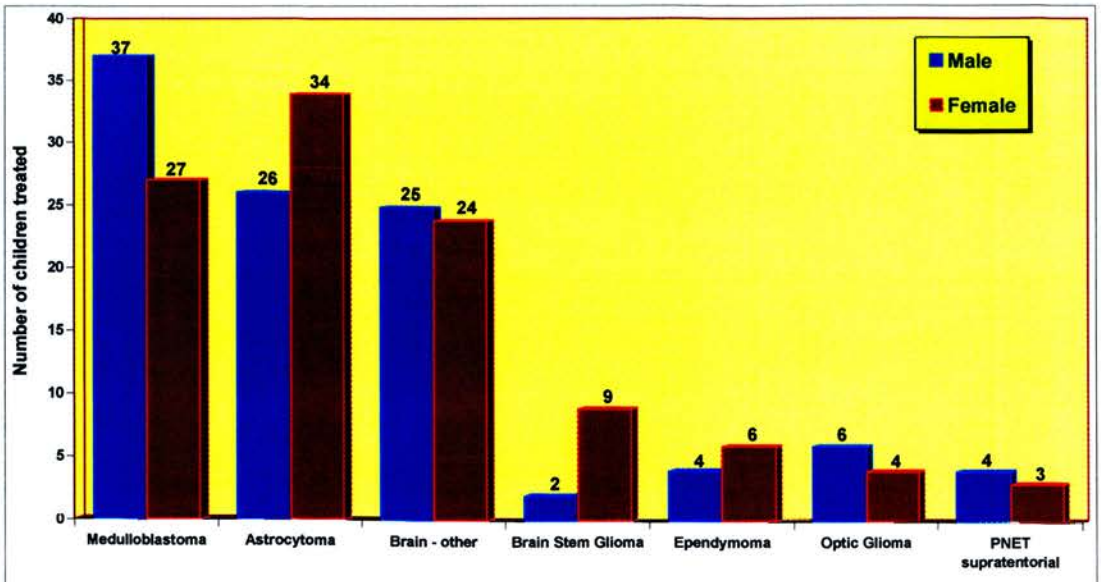

Figure 3.10. Diagnostic breakdown of children treated for brain tumours in Glasgow, 1975 to 1995.

(Brain – other includes rarer brain tumours including germ cell tumours, neuroectodermal tumours, dysgerminomas, germinomas, pineal tumours, pontine tumours and teratomas.)

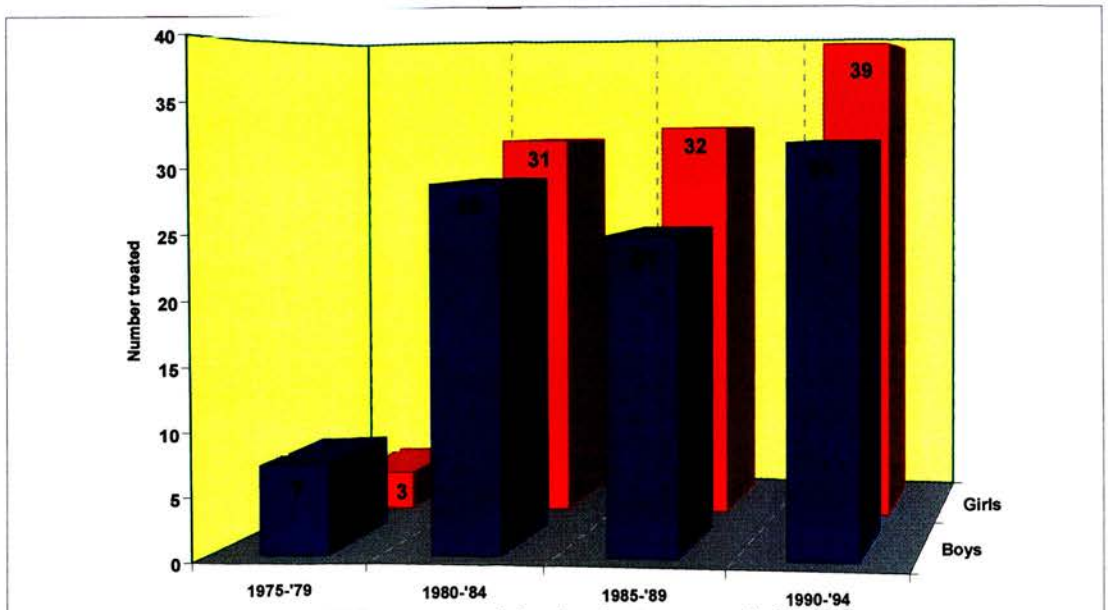

Figure 3.11. Number of children treated for brain tumours in RHSC, Glasgow (1975 to 1995).

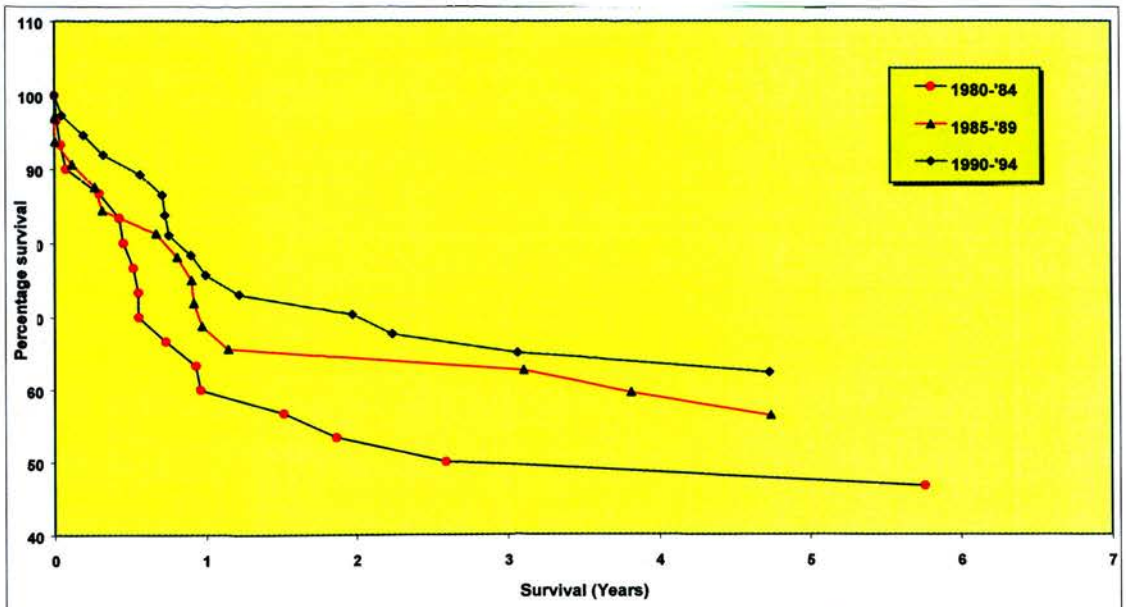

Figure 3.12. Survival rates for children with brain tumours in RHSC, Glasgow (1980 to 1994).

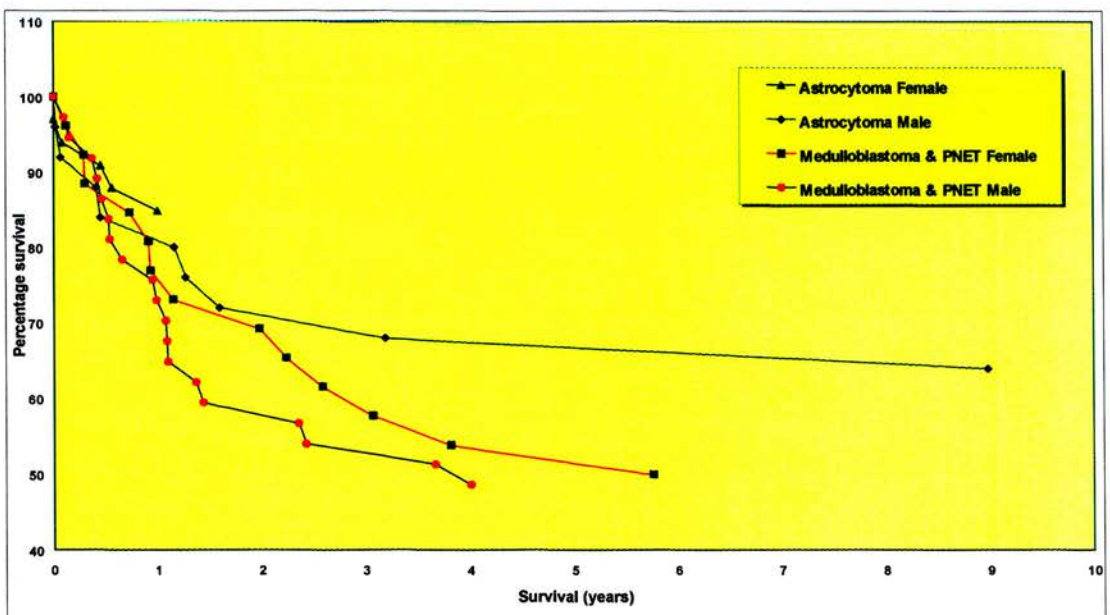

Figure 3.13. Overall survival curves according to type of brain tumour in RHSC, Glasgow (1980 to 1994).

3.2.2 Criteria for Patient Selection

Patients included in the study were those who were at least one year post completion of their anti-cancer treatment and who had received a sufficiently high dose of cranial irradiation to put them at risk of developing SAI. Patients included were those who at the time were willing to take part in the study. This meant that inclusion into the study was relatively arbitrary.

These included:

- Patients with ALL receiving cranial irradiation who then had acute relapse requiring further chemotherapy and/or cranial irradiation.
- Patients with brain tumours who had received high dose cranial irradiation (equal to or greater than 3000cGy) as part of their treatment.
- Patients who had received a bone marrow transplant either as the treatment of their initial disease, (e.g. acute myeloid leukaemia, stage four neuroblastoma), or who had received a bone marrow transplant for relapse of their initial disease (e.g. ALL).
- Patients already on steroid replacement for suspected hypoadrenalism were included in the study if the diagnosis of SAI was considered on review to be inconclusive.

3.2.3 Exclusion Criteria

Patients who were not included in the study were those with ALL in first remission who had received standard ALL treatment - two years chemotherapy and cranial irradiation, 1800cGy. Also excluded were children with other leukaemias treated with chemotherapy only, children with solid tumours out with the hypothalamo-pituitary axis which were not treated with radiotherapy, and children in whom the diagnosis of

SAI was considered so likely that discontinuation of their steroid therapy might be hazardous.

Patients were also excluded from the study if patients or their parents requested not to be entered.

3.2.4 Auxology

The study patients' growth and development were monitored regularly in the out-patient clinics at the Royal Hospital for Sick Children, Glasgow. The patients' height was measured by trained auxiliary staff using fixed wall-mounted stadiometers, which were regularly calibrated.

Patients were weighed using electronic sitting scales.

3.2.5 Collection of salivary and capillary samples in study patients

3.2.5.1 Salivary samples

Saliva samples were collected at home in the same way as in the control subjects. The children were asked to collect samples when they were well and when they were unwell.

3.2.5.2 Capillary samples

Capillary blood spot samples were collected from the study patients but not the controls. Capillary blood was taken from the finger immediately after collecting saliva samples, using an Autolet lite capillary blood sampling device. Blood was collected onto filter paper strips and left to dry at room temperature.

3.2.6 Low-dose Synacthen Test

A low dose Synacthen test was performed in study patients who had either not had anterior pituitary function tests, or in those who had had an insulin tolerance test more than one year previously.

Patients who had had an ITT performed more recently than this, also had a low dose Synacthen test if it was felt they had features, which were suggestive of SAI.

A low dose synacthen test was performed according to a previously published protocol (Crowley, Hindmarsh, Holownia, Honour, & Brook 1991). Synacthen (500ng/1.73m^2 body surface area) was administered intravenously and the response assessed by measuring plasma cortisol concentrations from plasma samples taken at intervals over 35 minutes (See appendix 4 for detailed protocol).

3.2.7 Insulin Tolerance Test

An insulin tolerance test (ITT) was performed as part of the routine test to assess anterior pituitary function. These tests are performed routinely in RHSC at least one year following completion of anti-cancer treatment in those children at risk of developing endocrine deficit. The ITT was performed by giving short acting insulin in a dose of 0.1 or 0.15 units/kg intravenously, and measuring blood glucose, growth hormone and cortisol over the following 2 hours. A TRH test and LHRH test was performed at the same time (see appendix for detailed protocol).

3.2.8 Single Dose Oral 3 Day Urinary Metyrapone Test

A urinary metyrapone test was performed in five patients by giving metyrapone in a dose of 500 mg/m^2 body surface area, collecting urine for 24 hours before administration, and then for three 24 hour periods thereafter. Urinary steroid profiles were performed using capillary column gas chromatography/mass spectrometry (See Appendix 7 for protocol).

4 Materials and Methods used in Assay Development

4.1 Apparatus

The apparatus listed below were used in the assay development:

- A NE-1600 Gamma Counter (Nuclear Enterprises, Edinburgh, Scotland) was used to measure gamma radiation.
- Centrifugation was carried out using a M.S.E. Coolspin 2 centrifuge (Medical Scientific Equipment, Manor Road, Crawley, West Sussex, England).
- Borosilicate glass disposable culture tubes were used; size 12x75 mm and 16x100 mm.
- Plastic tubes (5 ml and 2.5 ml size) were used for storing samples and standards at -20°C. Disposable sarstedt pipette tips were used throughout. These were obtained from Sarstedt Ltd., Beaumont Leys, Leicester, England.
- Oxford sampler pipettes (50 µl,100 µl, 200 µl, 500 µl, 1 ml) were used throughout for pipetting samples and standards.
- Repeat pipetting was performed using a BCL repeating pipette (Boehringer Corporation Limited, Bell Lane, Lewes, East Sussex, England).
- An SMI Digitron electronic pipette (Sherwood Medical Industries Limited, County Oak Way, Crawley, East Sussex, England) was used to pipette small samples of label for dilution.

- For solvent extraction, an S.M.I. Multi-tube Vortexer from Alpha Laboratories, Easteigh, Hampshire, England, and a Buchler Vortex evaporator from Arnold Horwell, London, England, were used.
- Filter paper for blood spot strips (code 31 ETCHR; catalogue number 3031.915) was obtained from Whatman International Ltd., Maidstone Kent, England.
- A Watson-Marlow pump was used for suctioning the supernatant following washing and centrifuging test tubes (obtained from Falmouth, Cornwall, England).
- Data analysis was performed on an Apple computer using the WHO program for Radioimmunoassays, by P.R. Edwards (Department of Nuclear Medicine, Middlesex Hospital, London, England).
- For measuring control children, a free standing stadiometer was used (Raven Equipment, Castlemead Equipment, 12 Little Mundells, Welwyn Garden City, Hertfordshire, AL7 1EW).
- For measuring the study group, a fixed wall-mounted Harpenden stadiometer was used (Holtain, Dyfed, Wales).
- Soehnle electronic weighing scales were used for weighing the control group (CMS weighing Equipment Limited, 18 Camden High Street, London, NW1 0JH).

4.2 Reagents

- All common reagents and solvents were of 'AnalaR' grade and were from either B.D.H. Chemicals Ltd. or Sigma Chemical Co., Dorset, England.
- Steroids were obtained from Sigma Chemical Company.

- Hexane and diethyl ether were obtained from Rathburn Chemicals Ltd., Walkerston, Scotland.
- Newly expired red blood cells were obtained from Blood Transfusion Service, Glasgow Royal Infirmary, Glasgow, Scotland.
- Sheep anti-cortisol antibody was obtained from the sheep antibody production unit (SAPU), Scottish blood transfusion unit, Law hospital, Scotland.
- Sheep anti-cortisol antibody (code number HP/S/631-2C) was purchased from Guildhay Antisera, University of Surrey, England.
- Sheep anti-cortisol antibody (code number so20 lot M421) was purchased from Guildhay Antisera, University of Surrey, England.
- 17-hydroxyprogesterone antibody was raised in-house by A.M. Wallace. Rabbit antiserum was raised against 17-hydroxyprogesterone-3-carboxymethyloxime-bovine serum albumin (code W2B). This was microencapsulated (by A.M. Wallace) before use (Wallace & Wood 1984).
- Androstenedione antibody was raised in-house by A.M. Wallace (Thomson et al 1989). Rabbit antiserum was raised against androstenedione-3-carboxymethyloxime-bovine serum albumin.
- Cortisol radio-label (cortisol-3-carboxymethyloxime[^{125}I]-iodohistamine) was prepared in-house, or purchased from Amersham International plc., Lincoln Hall, Green End, Aylesbury, Buckinghamshire, England (Code IM129).
- 17-hydroxyprogesterone radio-label (17-hydroxyprogesterone-3-carboxymethyloxime[^{125}I]-iodohistamine) was prepared in-house.
- Androstenedione radio-label (Androstenedione-3-carboxymethyloxime[^{125}I]-iodohistamine) was prepared in-house.

- Encapsulated antibody - Permeable Antibody-containing Microcapsules (PAM)

Anti-cortisol antibody encapsulated in semipermeable nylon microcapsules in the form of a suspension (by A.M. Wallace) before use (Wallace & Wood 1984).

PAM 251 - SAPU cortisol antibody, code no.

PAM 253 - Cardiff cortisol antibody, code no. R9B4.

PAM 254 - Guildhay cortisol antibody (400 μl), code no. HP/S/631-2C.

PAM 256 - Guildhay cortisol antibody (100 μl), code no. HP/S/631-2C.

PAM 257 - Guildhay cortisol antibody (100 μl), code no. HP/S/631-2C.

PAM 258 - Guildhay cortisol antibody (100 μl), code no. S020 lot M421.

4.2.1 Calculations

Area under the curve was calculated using the equation:

$(t^2-t^1)(y^1+y^2)+(t^3-t^2)(y^2+y^3)+(t^4-t^3)(y^3+y^4)+(t^5-t^4)(y^4+y^5)+(t^6-t^5)(y^5+y^6)$

where t corresponds to the time the sample was taken in hours, y corresponds to the concentration of steroid hormone at the corresponding time (Matthews et al 1990).

The equation was adjusted according to the number of samples taken over 24 hours. If there were less than five samples in 24 hours, then area under the curve was not calculated.

Statistical calculations were performed using the Minitab program (version 9.0; Clecom microcomputer specialists, The Research Park, Edgbaston, Birmingham).

4.2.2 Solutions and Buffers

The following solutions and buffers were used in the salivary and capillary blood spot assays.

4.2.3 Phosphate Buffer

Phosphate buffer (0.05M, pH 7.4), was used as a basic radioimmunoassay buffer for the salivary steroid assays. It was made by mixing two solutions together:

Solution A (0.25M sodium dihydrogen orthophosphate solution)

This was prepared by dissolving 78 g $NaH_2PO_4.2H_2O$ in distilled water and made up to two litres.

Solution B (0.5M disodium hydrogen orthophosphate solution)

This was prepared by dissolving either:

142 g Na_2HPO_4 (anhydrous)

358 g $Na_2HPO_4.12H_2O$

178 g $Na_2HPO_4.2H_2O$

in distilled water and making up to two litres.

Solution A (30 ml) was added to solution B (120 ml) and mixed.

Sodium Chloride (17.5 g) was dissolved in 250 ml of distilled water. This was added to the above mixture and the solution made up to two litres with distilled water. The pH was checked and adjusted to pH 7.4 by adding either acid (hydrochloric acid) or alkali (sodium hydroxide solution) as appropriate.

4.2.4 Salivary Assay Buffer

This was prepared by adding sodium azide (500 mg/L) and gelatine (1 g/L) to the phosphate buffer.

The solution was stored at $4^{o}C$ for up to 6 months.

4.2.5 Citrate/Phosphate Buffer

The citrate/phosphate buffer (pH 4.0), was used in the capillary blood spot cortisol assay. It was prepared by mixing two solutions together:

Solution A (0.1M citric acid)

This was prepared by dissolving 21 g citric acid in one litre of distilled water.

Solution B (0.2M disodium phosphate solution)

This was prepared by dissolving 35.6 g $Na_2HPO_4.2H_2O$ in one litre of distilled water.

1 g/L gelatin was then added to each solution.

For a solution of pH 4.0, 62 ml of solution (A) was added to 38 ml of solution (B).

The pH was checked and adjusted to pH 4.0.

The solution was stored at 4°C and kept for up to 6 months.

4.2.6 Isotonic Saline

Isotonic saline (0.9% NaCl w/v) was prepared by dissolving sodium chloride (9g) in one litre of distilled water.

4.2.7 Wash Solution

Wash solution (0.9% NaCl w/v, 0.1% gelatin w/v) was prepared by adding gelatin (1g) to each litre of freshly prepared isotonic saline and mixing thoroughly.

A fresh solution was prepared for each assay.

4.3 Preparation of Standards

4.3.1 Salivary Cortisol Standard Solutions

◊ A stock solution of 500 μmol/l of cortisol in ethanol was made by dissolving cortisol (18 mg) in ethanol (100 ml).

◊ The stock solution was diluted 10 times to give an intermediate solution of 50 μmol/L (1.8 mg/ml).

◊ An aliquot of 80 μl of the intermediate solution was placed in a volumetric flask. A volume of 125 ml of salivary assay diluent was added to give a working solution of 32 nmol/L (11.6 ng/ml).

◊ Working standards were prepared by serial dilution of the working solution (0; 0.13; 0.25; 0.5; 1; 2; 4; 8; 16; 32 nmol/L).

◊ Aliquots (200 μl) of each standard solution were pipetted into appropriately labelled plastic tubes, sealed and stored at -20°C.

4.3.2 Salivary 17-Hydroxyprogesterone Standards

◊ A stock solution of 3 μmol/L of 17-OHP (molecular weight 330.5) in ethanol was made by dissolving 17-Hydroxyprogesterone (1 mg) in ethanol (1 L).

◊ The stock solution was diluted 100 times to give a working solution of 30 nmol/L.

◊ Working standards were prepared by serial dilutions of the working solution (0; 0.15; 0.3; 0.6; 1.2; 2.4; 4.8 nmol/L).

◊ Aliquots (300 μl) of each standard solution were pipetted into appropriately labelled plastic tubes, sealed and stored at -20°C.

4.3.3 Salivary Androstenedione Standard Solutions

◊ A stock solution of 3.5 μmol/l of androstenedione (molecular weight 286.0) was made by dissolving 1 mg of androstenedione in 1 litre of ethanol. This is diluted 1000 times by adding 100 μl of stock solution to 100 ml of salivary assay diluent, to give a solution of 3.5 nmol/L.

◊ Working standards were prepared by serial dilutions of the working solution (0; 0.018; 0.035; 0.07; 0.14; 0.28; 0.56; 1.12 nmol/L).

◊ Aliquots (300 μl) of each standard solution were pipetted into appropriately labelled plastic tubes, sealed and stored at -20°C.

4.3.4 Capillary Blood Spot Cortisol Standard Solutions

◊ Using the original stock solution (500 μmol/L cortisol in ethanol), a solution of cortisol in steroid hormone stripped serum was made (2000 nmol/L) by adding 200 μl of stock solution to 50 ml of stripped serum. This was used as the working solution.

◊ Working standards of cortisol in stripped serum were then prepared by serial dilution of the working solution (0; 50; 100; 200; 300; 400; 800; 1200; 1600; 2000 nmol/L).

◊ Each of the above standard solutions is then double-diluted with washed red blood cells to obtain the required blood spot standards (0; 25; 50; 100; 150; 200; 400; 600; 800; 1000 nmol/L).

4.3.5 Preparation of washed red blood cells

◊ Newly expired packed erythrocytes (160 ml) were divided equally into four graduated polypropylene centrifuge tubes.

◊ The tubes were centrifuged at 3000 r.p.m. for 20 minutes at 4°C.

◊ The top layer of serum and erythrocytes was then aspirated, leaving 25 ml of packed erythrocytes in each tube.

◊ Isotonic saline (25 ml) was added to each tube. This was mixed well and centrifuged again.

◊ The supernatant was again aspirated and discarded.

◊ The red cells were re-washed with saline and centrifuged once more.

◊ The supernatant and the top layer of erythrocytes was aspirated once more, leaving 15 ml of packed erythrocytes in each tube.

◊ The aliquots of packed erythrocytes were pooled and mixed.

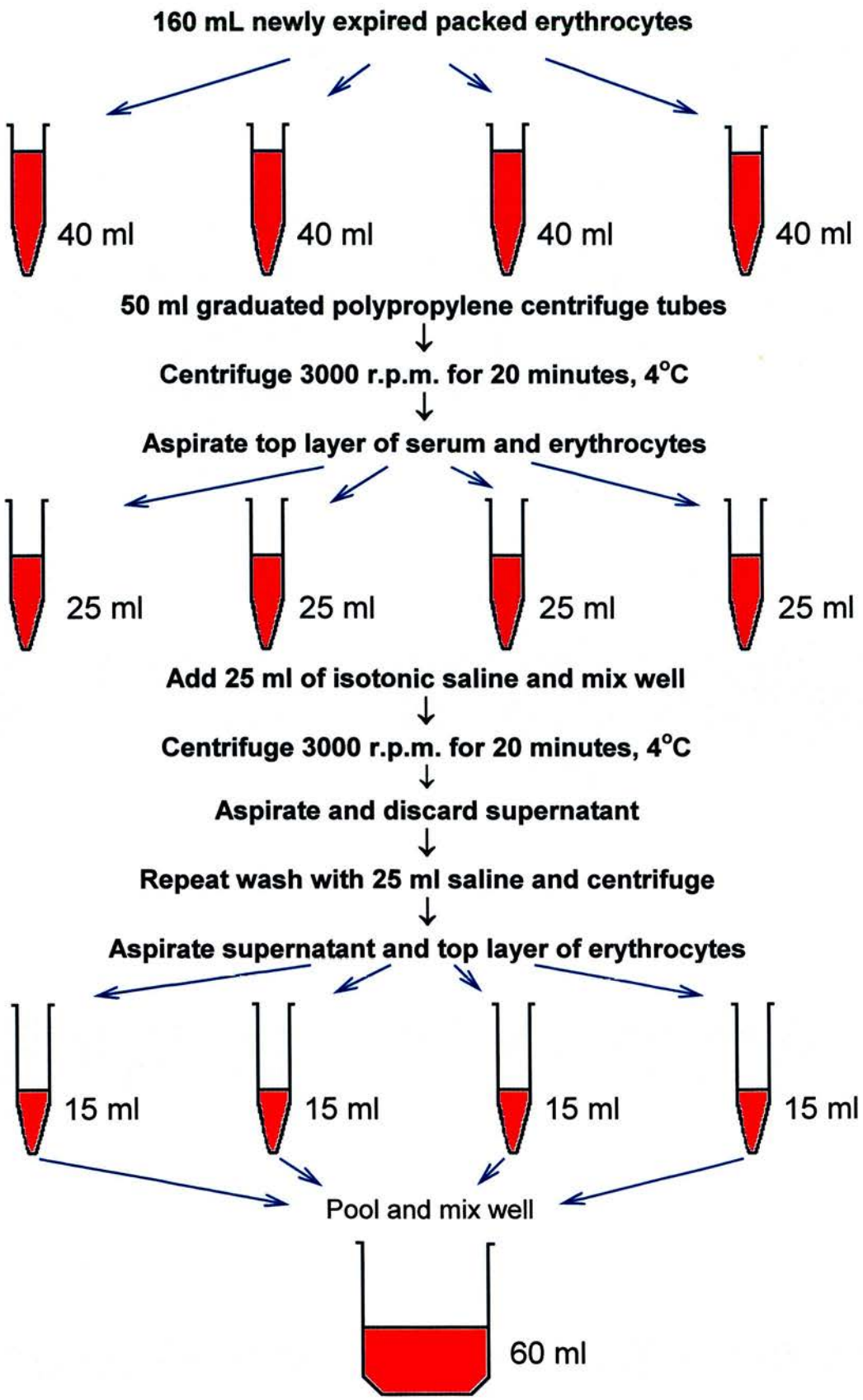

Figure 4.1. *Preparation of washed red blood cells.*

4.4 Salivary Steroid Assays

The salivary cortisol, 17-OHP and androstenedione assays were performed using the following common reagents, unless otherwise stated.

4.4.1 ^{125}I radiolabel

The radiolabel was potency checked and the solution diluted to 10,000 counts per minute in salivary assay diluent.

4.4.2 Antibody preparation

Permeable Antibody-containing Micro-capsules (PAM) were used in the salivary cortisol assay (PAM 258), and in the salivary 17-OHP assay (PAM 259).
The salivary androstenedione assay employed a double antibody method.

4.4.3 Quality controls

Samples of saliva provided by healthy adult volunteers at various times of the day were used as quality control samples. The samples were stored at -20°C and thawed prior to use in the same way as the patient samples.

4.5 Salivary Cortisol Assay

4.5.1 Assay Procedure

◊ All saliva samples were collected in clean dry plastic specimen containers and frozen to -20°C. This denatured the glycoprotein.
The samples were thawed to room temperature and centrifuged at 2000 r.p.m. for 5 minutes. The clear supernatant was used for the assay.

◊ Each standard (50 μl) and saliva sample (50 μl) was pipetted into duplicate labelled tubes in the same way.

- ◊ ^{125}I- labelled cortisol (100 μl at 10,000 c.p.m./tube) was added to each tube. Total count tubes contained 100 μl of this reagent only.
- ◊ Well-mixed PAM 258 antibody (200 μl of 1 in 50 dilution) was added to each tube, except the total count tubes and non-specific binding (N.S.B.) tubes.
- ◊ Duplicate N.S.B. samples in labelled tubes consist of 50 μl of assay diluent, 100 μl of ^{125}I-cortisol, and 200 μl of PAM 247 (anti-thyroid antibody).
- ◊ All tubes were vortex mixed and left to incubate at room temperature for 1½ hours.
- ◊ The total count tubes were put aside.
- ◊ All other tubes were washed in wash solution, using the Watson-Marlow semi-automated washing apparatus, then centrifuged at 2000 r.p.m. for 5 minutes. This was repeated once (total of two times).
- ◊ All tubes were counted using a multi-well gamma counter NE-1600. The results were then calculated using the W.H.O. program for radioimmunoassay and charted on log scale.

The method is illustrated in Figure 4.2.

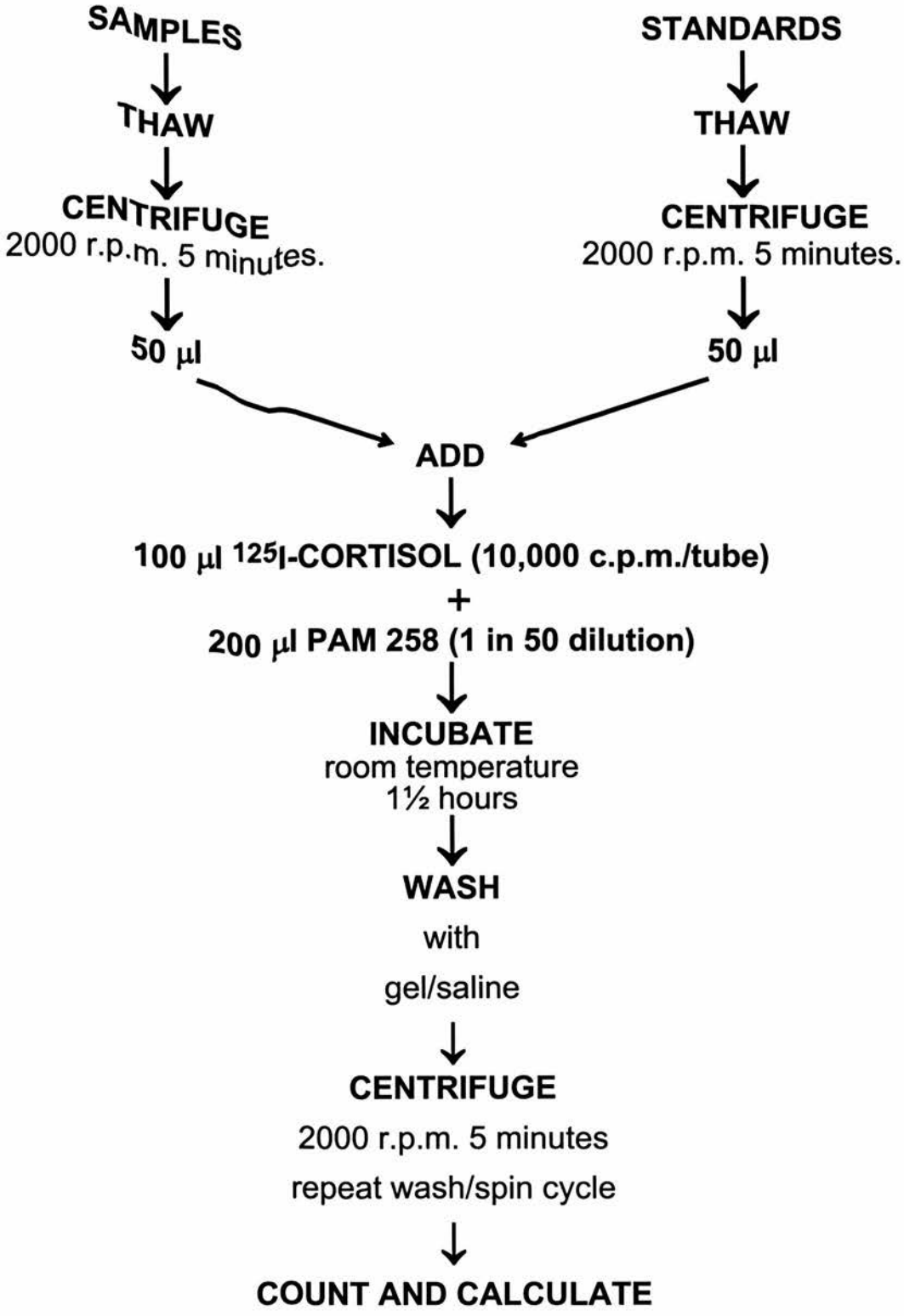

Figure 4.2. ***Summary of procedure for radio-immunoassay of cortisol in saliva.***

4.6 Salivary 17-Hydroxyprogesterone Assay

4.6.1 Assay Procedure

◊ The procedure for assay of 17-OHP was similar to the cortisol assay, except for the following:

^{125}I- labelled 17-OHP (100 μl of 10,000 c.p.m./tube) was used.

Well-mixed PAM 259 antibody (200 μl of 1 in 50 dilution) was used.

Incubation time was 45 minutes.

The method is illustrated in Figure 4.3.

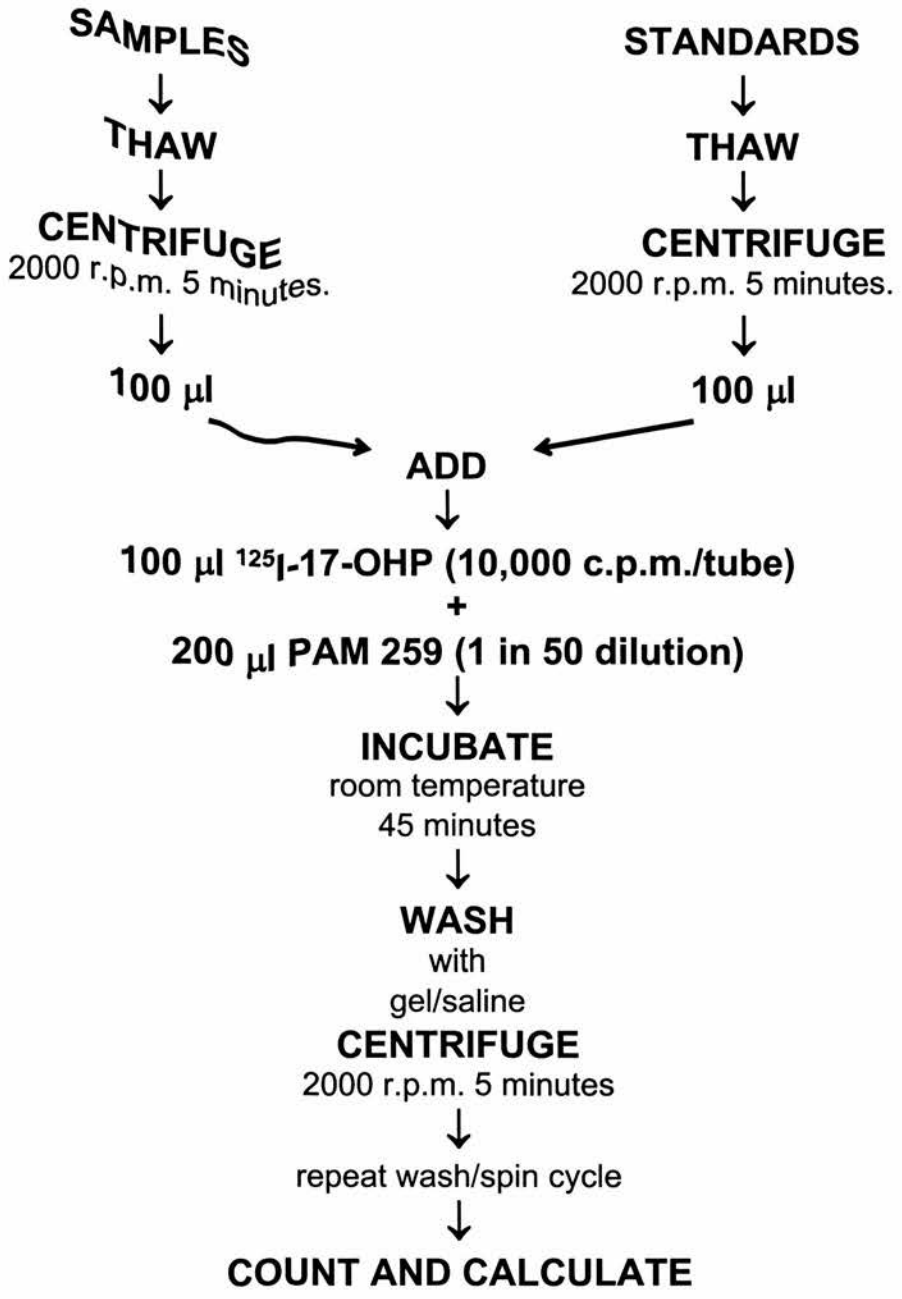

Figure 4.3. *Summary of procedure for radioimmunoassay of 17-hydroxyprogesterone in saliva.*

4.7 Salivary Androstenedione Assay

4.7.1 Anti-androstenedione antibody.

First antibody - rabbit A4-3-CMO-BSA (1 in 200,000).
(50 μl of antibody, pre-diluted to 1 in 2,000, in 50 ml of assay diluent.)

Second antibody - donkey anti-rabbit antiserum (D.A.R.) with N.R.S. (normalised rabbit serum) added.
(1 ml of D.A.R., 80 μl NRS in 20 ml of assay diluent.)

4.7.2 Assay Procedure

◊ All saliva samples were collected in clean dry plastic specimen containers and frozen at -20°C.
The samples were thawed at room temperature and then centrifuged at 2000 r.p.m. for 5 minutes. The supernatant was used for the assay.

◊ Each standard and saliva sample (100 μl) were transferred into duplicate labelled tubes, using a manual pipette and Oxford tips.

◊ Hexane-ether (3 ml of 4:1 mixture v/v) was added to the tubes and vortex-mixed in a multi-tube vortexer for four minutes.

◊ The lower aqueous phase of the mixture was then snap frozen by immersing the lower part of the test tube in a bath of solid carbon dioxide and methanol.

◊ The upper hexane:ether phase was carefully decanted into a glass tube. This phase contains the extracted steroid.

◊ The solvent was then evaporated from all the tubes in a Buchler Vortex evaporator.

◊ ^{125}I- labelled androstenedione (100 μl of 10,000 c.p.m./tube) was added to each tube.

Total count tubes contained 100 μl. of this reagent only.

◊ First antibody (200 μl) was added to each tube, except the total count tubes and non-specific binding (N.S.B.) tubes.

◊ All tubes were vortex mixed and left to incubate at room temperature for at least 3 hours.

◊ Second antibody (400 μl) was added to each tube. The mixture was again vortex mixed and left to incubate overnight at 4°C.

◊ The total count tubes were put aside. All other tubes were centrifuged at 3000 r.p.m. for 60 minutes. The supernatant was then aspirated from all tubes, leaving the precipitate.

◊ All tubes were counted using a multi-well gamma counter NE-1600. The results were then calculated using the W.H.O. program for radioimmunoassay and charted on log scale.

The method is illustrated in Figure 4.4.

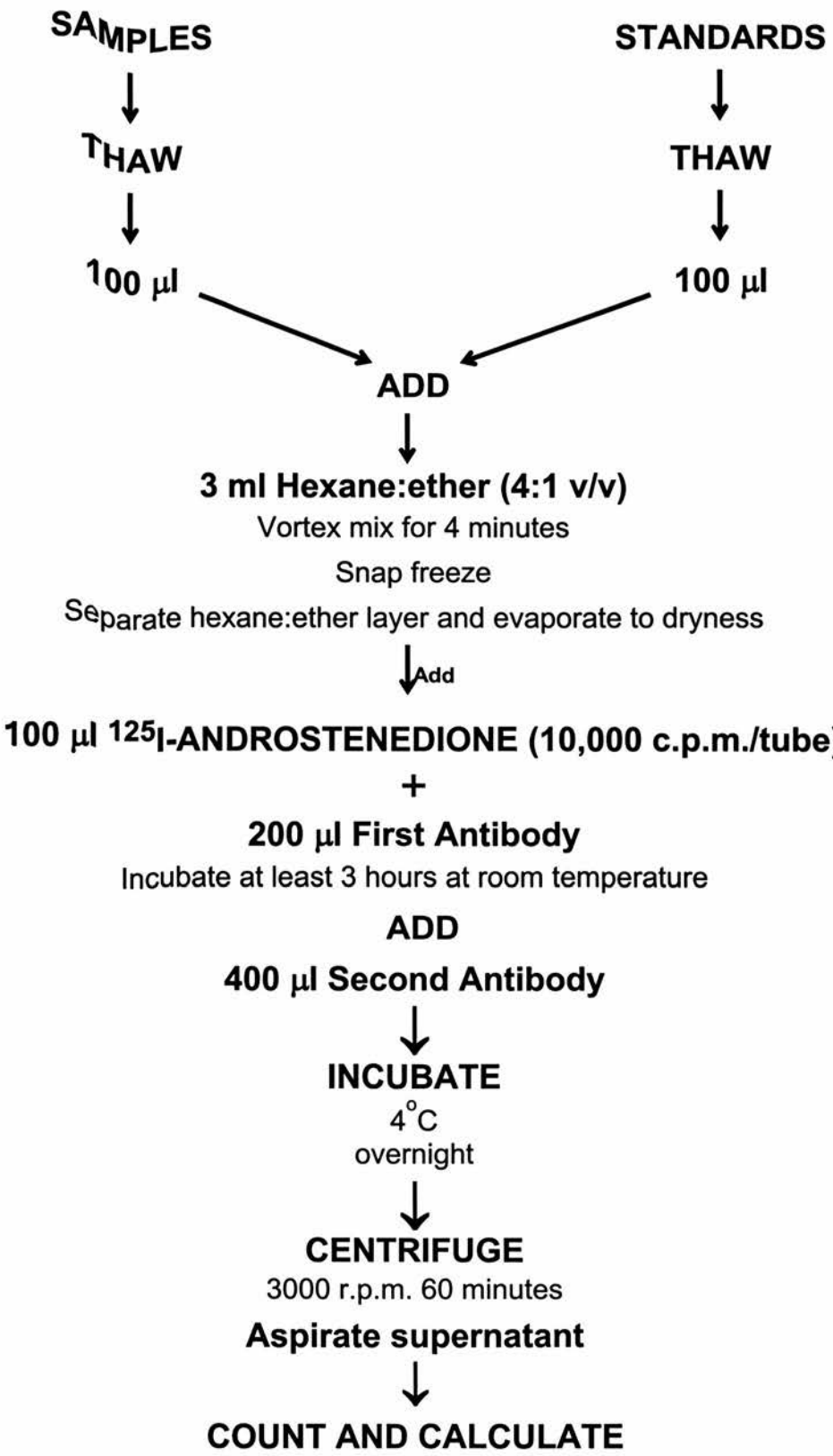

Figure 4.4. *Procedure for radioimmunoassay of androstenedione in saliva.*

4.8 Capillary Blood Spot Steroid Assays

The blood spot cortisol, 17-OHP and androstenedione assays were performed using the following common reagents, unless otherwise stated.

4.8.1 ^{125}I radiolabel

The radiolabel was potency checked and the solution diluted to 10,000 counts per minute in salivary assay diluent.

4.8.2 Antibody preparation

Permeable Antibody-containing Micro-capsules (PAM) were used in the blood spot cortisol assay (PAM 258), and in the blood spot 17-OHP assay (PAM 259). The antibodies were stored at 4°C.

The blood spot androstenedione assay employed a double antibody method.

4.8.3 Quality controls

Capillary blood spot samples provided by healthy adult volunteers at various times of the day were used as quality control samples. The samples were stored at room temperature and used in the same way as the patient samples.

4.9 Capillary Blood Spot Cortisol Assay

4.9.1 Anti-cortisol antibody - stored at 4 ^{o}C

PAM 258 - 1 in 50 dilution.

4.9.2 Assay Procedure

◊ All capillary samples were collected on filter paper and stored at room temperature. All test tubes were prepared in duplicate and labelled.

◊ Two 2.5 mm diameter discs (equivalent to 6 μl of whole blood) were punched out of the filter paper with standard solutions. Citrate/phosphate buffer (100 μl) was added as eluting fluid.

◊ Capillary blood spot samples were punched into labelled paired tubes in the same way, and 100 μl of citrate/phosphate buffer is added to each tube.

◊ ^{125}I- labelled cortisol (100 μl of 10,000 c.p.m./tube) was added to each tube. Total count tubes contained 100 μl of label only.

◊ Well-mixed PAM 258 (200 μl of 1 in 50 dilution) was added to each tube, except the total count tubes and non-specific binding (N.S.B.) tubes.

◊ Duplicate N.S.B. samples in labelled tubes consist of 100 μl of citrate/phosphate buffer and a punched out disc of blank filter paper. 100 μl of ^{125}I-cortisol, and 200 μl of PAM 247 (anti-thyroid antibody) used as N.S.B.

◊ All tubes were vortex mixed and left to incubate at room temperature overnight.

◊ The total count tubes were put aside.

All other tubes were washed with a solution of gel in saline, using the Watson-Marlow semi-automated washing apparatus, then centrifuged at 2000 r.p.m. for 5 minutes. This was repeated once (total of two times).

◊ All tubes were counted using a multi-well gamma counter NE-1600.

The results were then calculated using the W.H.O. program for radio-immunoassay and charted on log scale.

The method is illustrated in Figure 4.5.

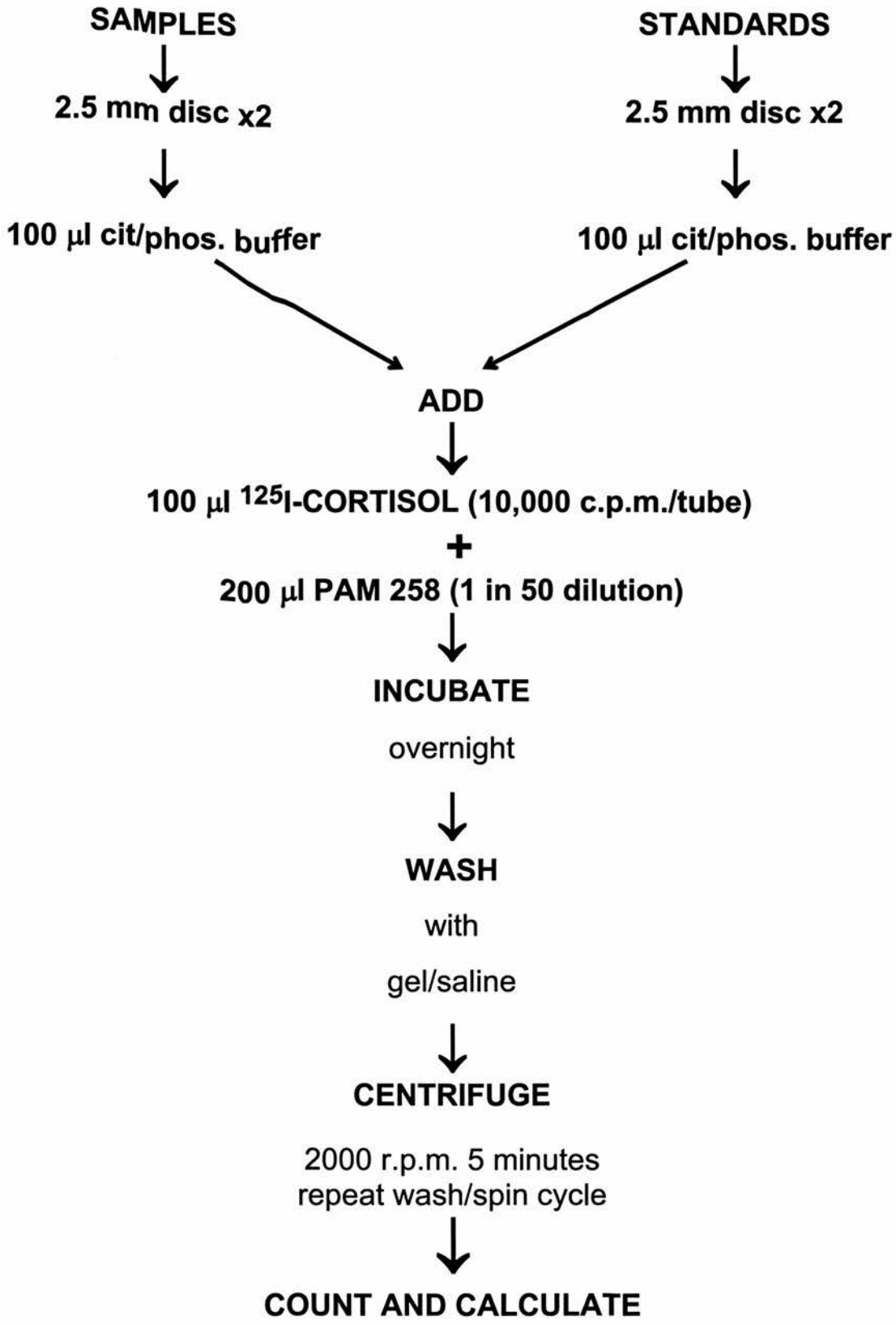

Figure 4.5. *Procedure for radio-immunoassay of cortisol in capillary blood spot samples.*

5 Development of Steroid Assays

5.1 Introduction

The aims of the project were to develop assay procedures for measuring steroids in saliva, a facility not at that time available in the regional endocrine laboratory. The three steroids to be assayed were cortisol, 17-hydroxyprogesterone (17-OHP) and androstenedione. The assay procedures needed to be relatively simple to perform, requiring minimal training. Ideally they would have a relatively rapid turnover time since they were to be used as a screening method for detecting low steroid levels in a large numbers of samples. They should also be sensitive and specific.

A capillary blood spot 17-OHP assay was already in use in the regional laboratory for monitoring patients with congenital adrenal hyperplasia. It was felt that this sensitive assay could be adapted for 17-OHP measurement in saliva. The same applied to a capillary blood spot androstenedione assay, which had recently been developed in the laboratory. By contrast a capillary cortisol assay was not established in the laboratory so that both blood spot and salivary cortisol assays needed to be developed.

5.2 Salivary Cortisol Assay Development

5.2.1 Introduction

The initial problem in developing the cortisol assays was the antibody selection. Initial experiments involved the use of a donkey anti-sheep cortisol antibody using a double antibody technique as a reference experiment. This was found to have a low sensitivity for the purposes of the study. The highly sensitive cortisol antibody developed by Chearskul (Chearskull 1985), was in short supply, so we had to find an alternative.

5.2.2 Antibody sensitivity and dilution in Salivary Cortisol Assays

The initial double antibody radioimmunoassay of salivary cortisol was performed using cortisol antibody from Scottish antibody production unit (SAPU). This antibody was available in reasonably large quantities and was used for the regional laboratory routine serum cortisol assay.

An antibody dilution curve was constructed using dilutions ranging from 1 in 50 to 1 in 204,800 were used. The assay was performed by adding 200 μl of cortisol label into duplicate labelled tubes. To each pair of tubes, 200 μl of antibody was added. After vortex mixing and incubating for two hours, donkey anti-sheep antibody (from SAPU) was added (400 ml) to each tube and incubated overnight at 4°C. The tubes were then centrifuged the following day at 3,000 r.p.m. for 45 minutes at 4°C. The supernatant was then removed by suction and the precipitates counted in a multi-well gamma counter (NE-1600). The results were then plotted on a chart (Fig. 5.1). The results showed that high concentrations of antibody inhibit antibody-cortisol binding, and with progressively lower antibody concentrations, the binding affinity increased, reaching a peak of 89% before falling exponentially.

The experiment was repeated by adding 1,000 nmol/L of cortisol in ethanol to each tube. The ethanol was evaporated to dryness before adding label and antibody. Antibody dilutions between 1 in 800 and 1 in 51,200 were used.

A Kelly curve was then constructed (Fig. 5.2). From this, an antibody concentration of 1 in 25,600 was found to give optimal results in terms of sensitivity.

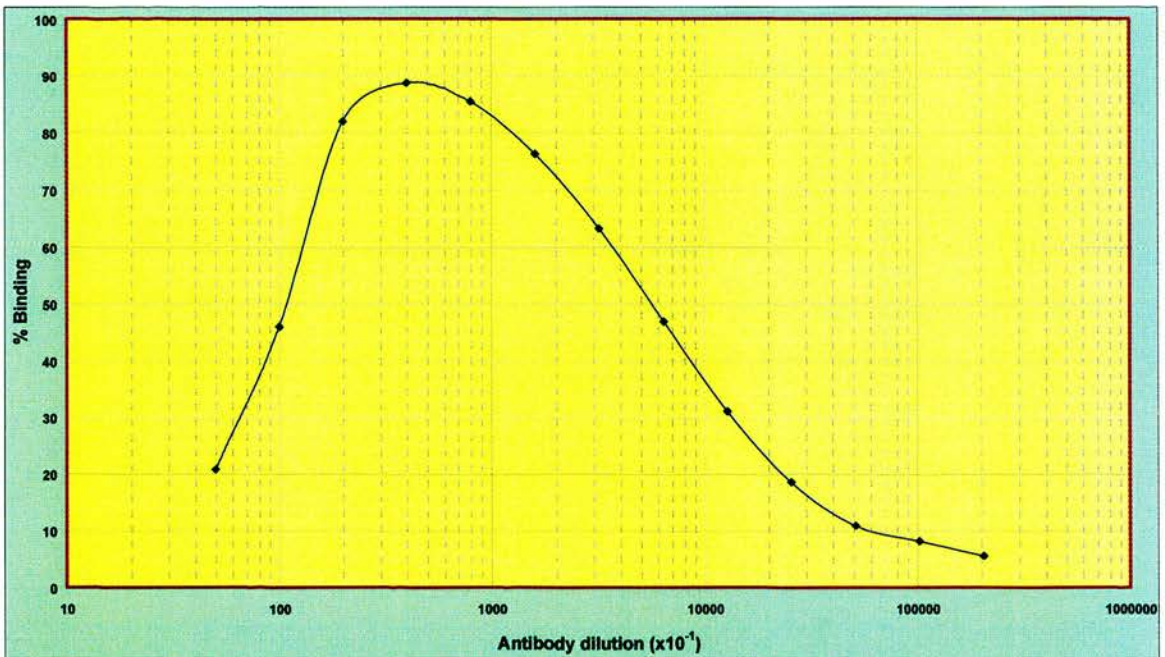

Fig. 5.1. Antibody dilution curve using SAPU antibody to determine optimal antibody dilution

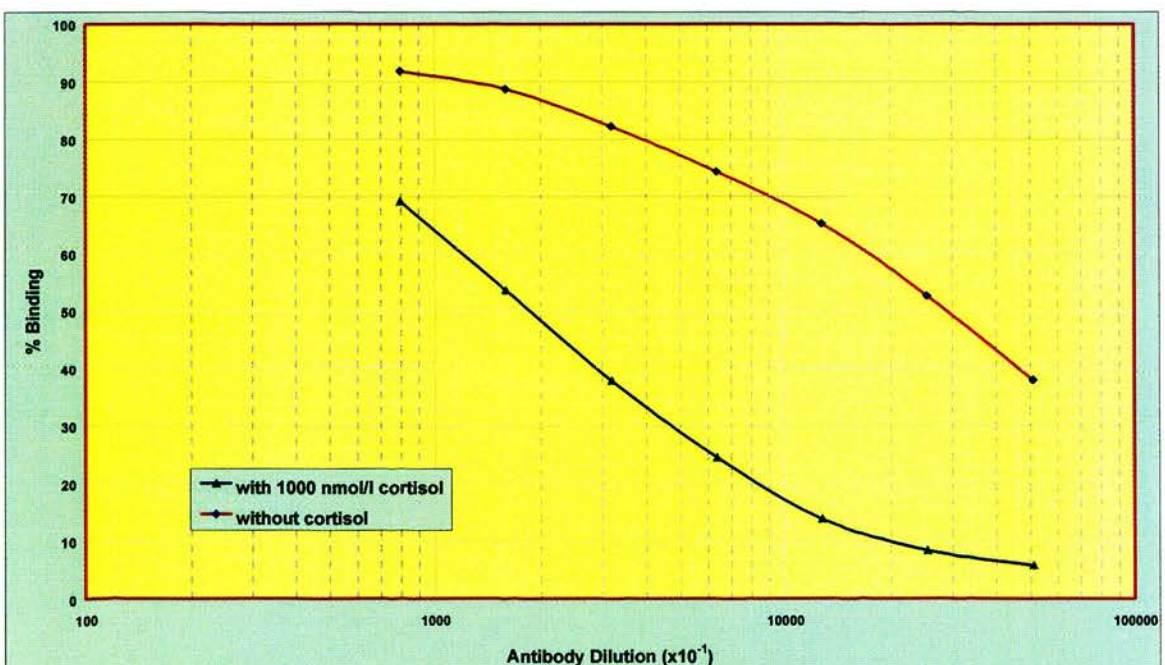

Fig. 5.2. Kelly curve using SAPU antibody; to determine the sensitivity range of the assay.

5.2.3 Standard Curve using Second Antibody Technique

Using the optimal antibody concentration described above, a standard curve was constructed for cortisol in ethanol. Concentrations from 0 to 1,000 nmol/L were used, including concentrations of 1.25, 2.5 and 10 nmol/L.

Each cortisol standard solution (50 μl) was added to paired tubes, and the ethanol evaporated off. The cortisol label and antibody were then added as before and the tubes incubated in the same way.

A standard curve of percentage binding against cortisol concentration was constructed. From this, the assay sensitivity was calculated to be 2.2 nmol/L (calculated by finding the concentration of cortisol required to produce a 10% drop in %B/Bo from 100% (at a concentration of 0 nmol/L).

5.2.4 Standard Curve using Capsulated Antibody

The donkey anti-rabbit antibody from SAPU was encapsulated (PAM 251) and a standard curve constructed. The cortisol antibody was encapsulated using the method by Wallace and Wood (Wallace & Wood 1984). Cortisol standards (range 0 to 1,000 nmol/L) were placed in duplicate tubes as before, and the ethanol evaporated off. Cortisol label (200 μl) was added to each tube along with capsulated antibody (200 μl). The tubes were vortex-mixed and left to incubate at room temperature for 90 minutes. The tubes were then washed in gel-saline and centrifuged at 2,000 r.p.m. for 5 minutes. The supernatant was aspirated, the wash cycle repeated, and the tubes, counted in a multi-well counter (NE-1600). The results were used to construct a standard curve. From this, the sensitivity of the assay was calculated, and found to be 1.7 nmol/L. This was more sensitive than the double antibody method. The use of PAM 251 in the assays was therefore developed further.

A salivary cortisol standard curve was then constructed in the same way as above, using PAM 251, but this time with cortisol standard concentrations ranged from 0 to 32 nmol/L. The standards were made in salivary assay diluent; 100 μl of each standard was placed in duplicate tubes, with 100 μl of cortisol label. The standard curves with ethanolic standards and salivary standards were compared. The standard curves were the same, indicating that cortisol-antibody binding was not affected by using salivary assay diluent.

In total, 10 assays were performed using PAM 251. The sensitivity for these assays was 1.2 nmol/L.

5.2.5 Comparison of Different Cortisol Antibodies for Salivary Assays

The levels of sensitivity obtained were felt to be too high for the purposes of this study. From the earlier work performed by Chearskull, (Chearskull 1985) it was known that PAM 253 (Cardiff antibody) was very sensitive, but unfortunately in short supply. The standard curve using this antibody was compared with PAM 251, and also with PAM 254, an antibody more readily available and in large supply.

Initial antibody dilution curves using 100μl volumes of standards suggested a dilution of 1 in 50 for PAM 253 and 1 in 100 for PAM 254. From the results, PAM 253 was found to give the most sensitive results, as expected. However, PAM 254 also gave acceptable results, with sensitivity below 1 nmol/L. The assay was therefore developed using this antibody (Figs. 5.3; 5.4; 5.5).

The assay sensitivity remained at 0.9 nmol/L in several assays performed (n = 15).

5.2.6 Comparison of Different Antibody Preparations and Concentrations

Standard curves using different antibody preparations were compared with PAM 253 and PAM 254. Two different preparations of Guildhay cortisol antibody were made. These were made by using lower concentrations of Guildhay antibody (400 μl and 100 μl) at the stage of encapsulation. These were named PAM 255 and PAM 256. The results of the standard curves were compared with PAM 253 and PAM 254. (Figs. 5.4; 5.5)The standard curve using PAM 256 was improved compared to the other standard curves.

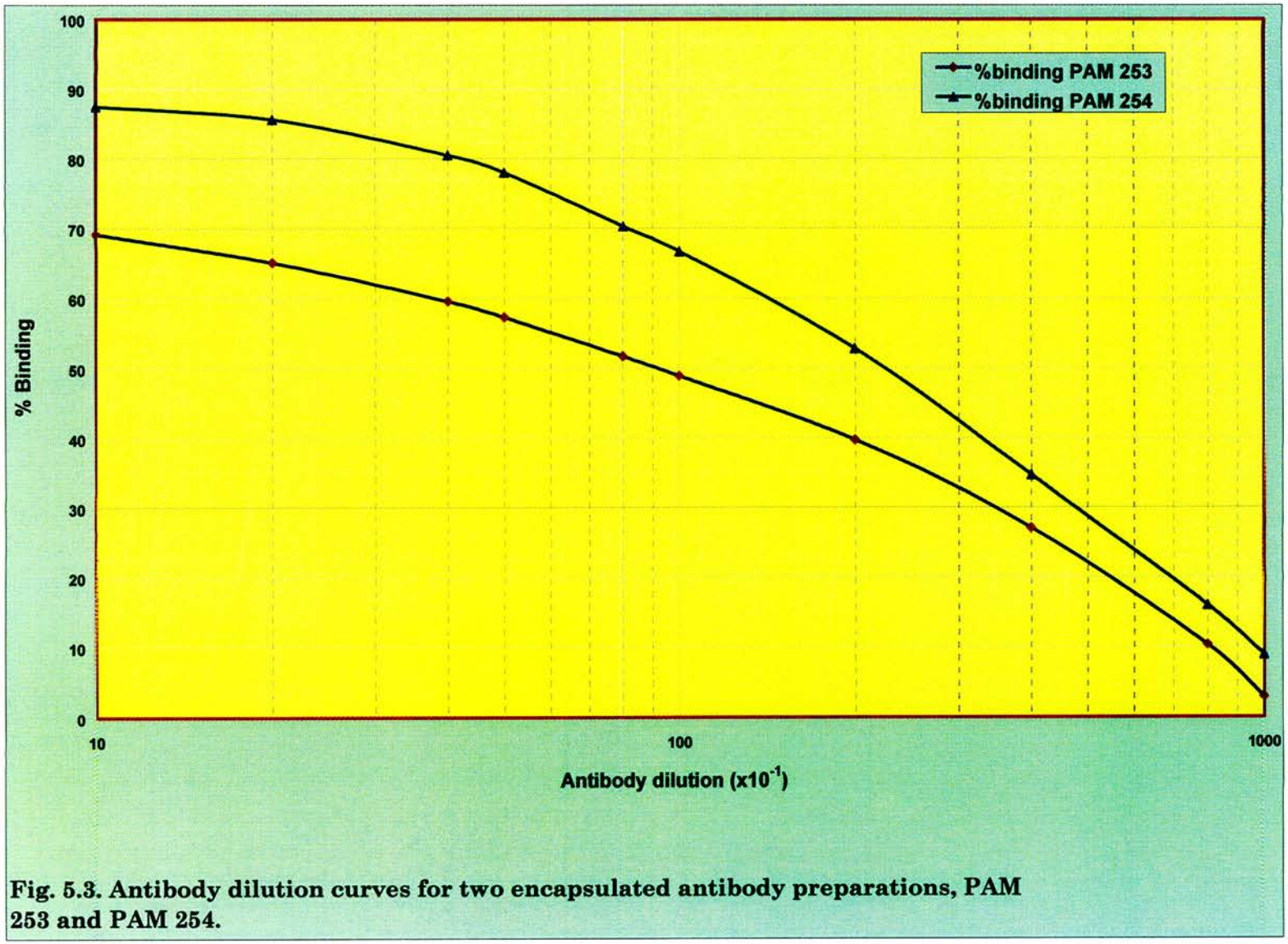

Fig. 5.3. Antibody dilution curves for two encapsulated antibody preparations, PAM 253 and PAM 254.

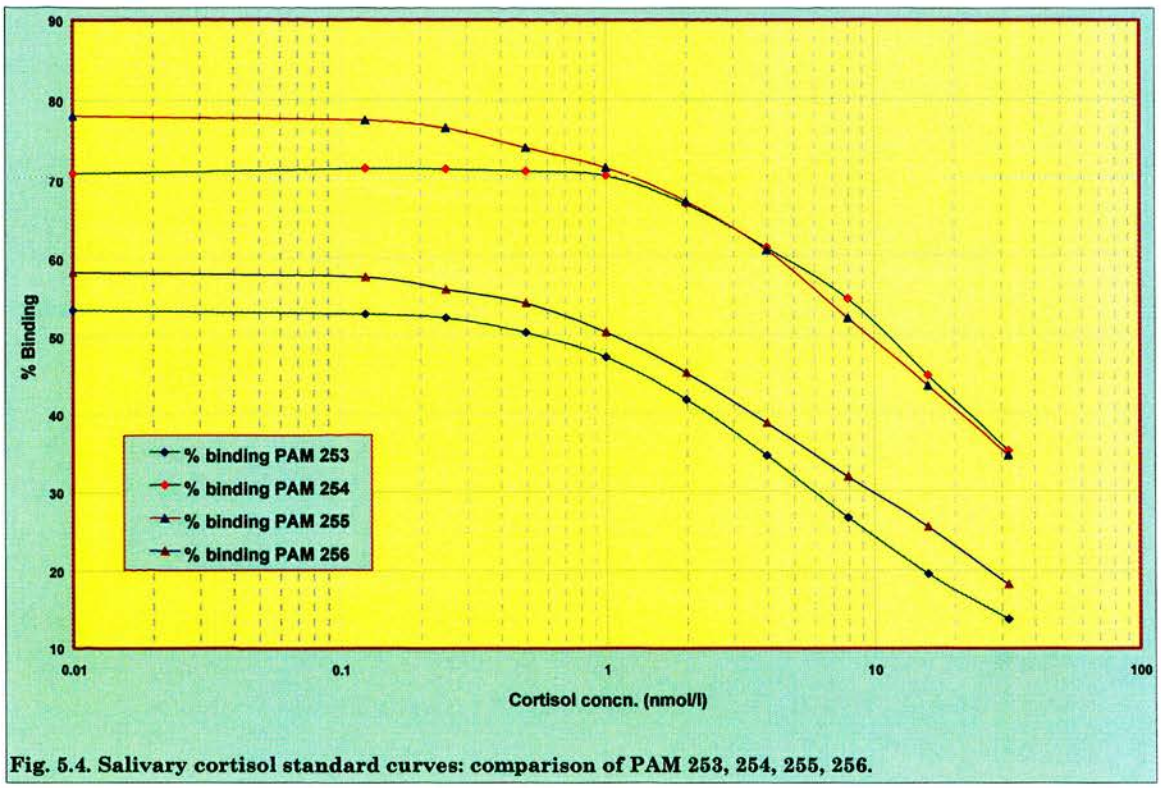

Fig. 5.4. Salivary cortisol standard curves: comparison of PAM 253, 254, 255, 256.

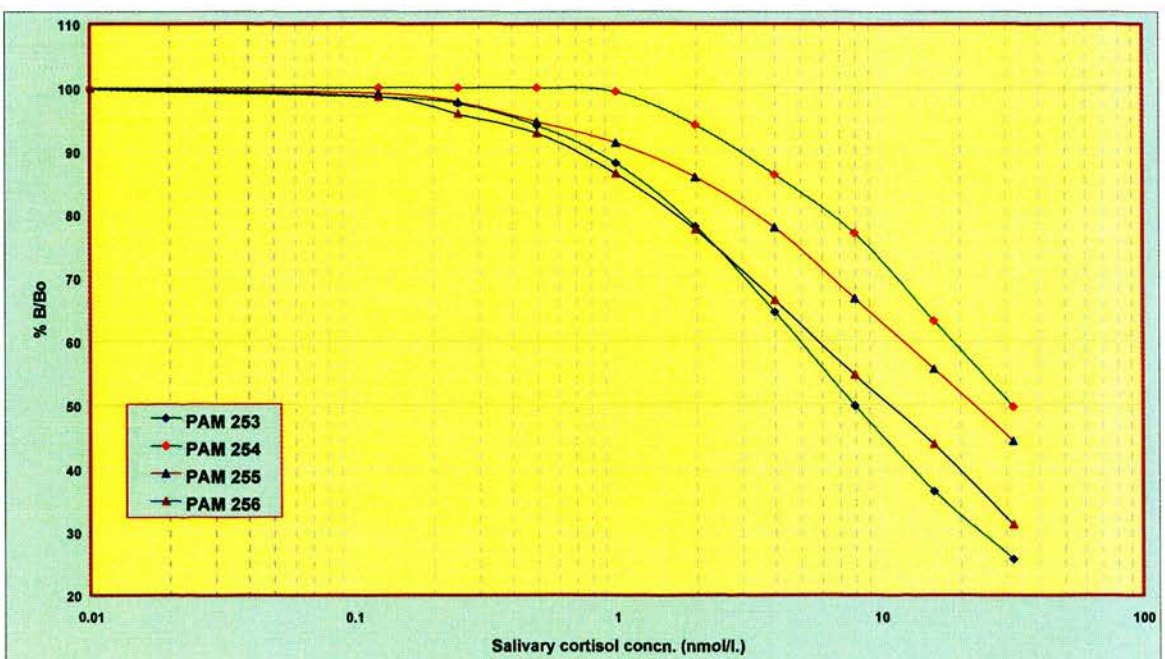

Fig. 5.5. Salivary cortisol standard curves for PAM 253, 254, 255, 256: %B/Bo against cortisol concentration.

5.2.7 Comparison of Different concentrations of Antibody and Standard Volumes

Using PAM 256 to construct standard curves, antibody dilutions of 1 in 50 and 1 in 100 were compared, together with sample volumes of 50 μl and 100 μl. An Antibody dilution of 1 in 100, and sample volume of 50 μl gave the best sensitivity and specificity. From a total of 15 assays, the sensitivity remained constant at 0.9nmol/L.

Unfortunately, when further supplies of antibody were obtained, antibody from a different bleed was provided. Further assays showed that the antibody sensitivity was different from the original work done. The assay sensitivity with PAM 258 was 1.5nmol/L. By repeating the standard curves with different antibody concentrations (PAM 257, PAM 258, made on separate occasions), it was found that the optimum antibody concentration was using PAM 258 at a dilution of 1 in 50. (Figs. 5.6; 5.7) The assay sensitivity with this antibody dilution was 0.9nmol/L, the same as the original sensitivity with the earlier encapsulated antibody.

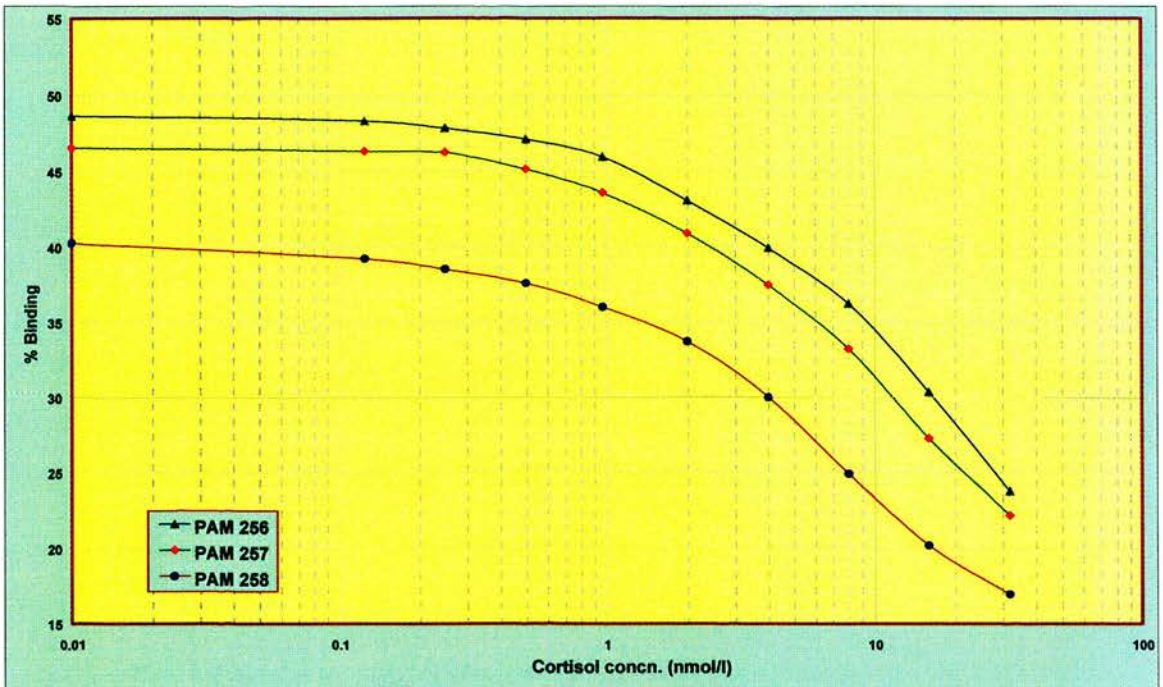

Fig. 5.6. Salivary Cortisol Standard Curve - Comparison of PAM 256, 257 and 258

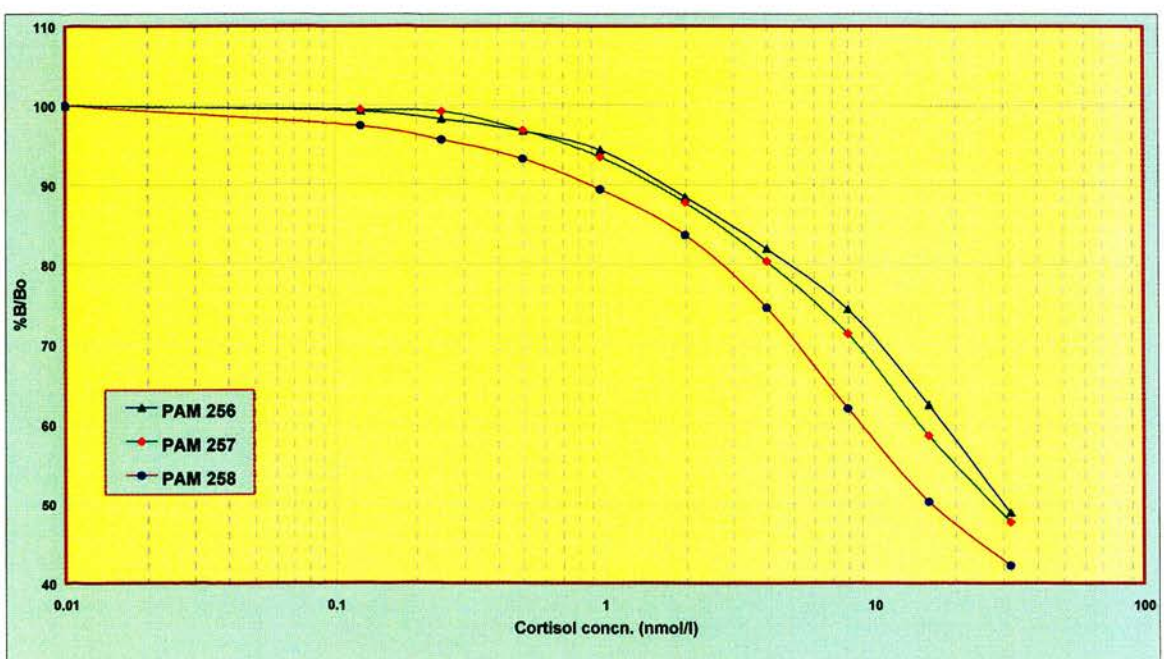

Fig. 5.7. Salivary cortisol standard curves - comparison of %B/Bo against cortisol concentration for PAM 256, 257, and 258.

5.2.8 Recovery Experiments for Salivary Cortisol Assay

Recovery experiments were performed by adding known amounts of cortisol in different concentrations (low and high) to samples of saliva of different concentration from healthy adult volunteers. Recovery of cortisol was calculated as a percentage by dividing the actual concentration measured by the expected concentration. The recovery of cortisol when low concentrations of cortisol was added was 96% and 113% in the low and high samples, and when high concentrations were added, the recovery was 111% and 94%.

5.2.9 Cross-reactivity Experiments

Cross-reactivity of cortisol to corticosterone, cortisone, prednisolone, 17-hydroxyprogesterone and 6-β-hydroxycortisol was determined in the salivary cortisol assay using PAM 258 antibody preparation using the method described by Abraham (Abraham 1969). These steroids were chosen in particular for two reasons: these steroids were important in saliva as they were present in significant amounts; the cross-reactivity figures of studies performed by the producers of the antibody suggesting that these steroid hormones produced significant cross-reaction with cortisol.

Each steroid was dissolved in ethanol to give a stock solution of 100μg/ml (1mg of steroid was dissolved in 10ml of ethanol). Following this, 1 ml of the stock standard solution for each steroid was diluted with ethanol (99 ml), to give an intermediate solution of 1μg/ml. These solutions were stored at 4°C until needed. Dose response curves were then derived for each competing steroid and compared with the cortisol dose response curve.

The standard curves were constructed and the mass of each competing steroid needed to displace 50% of the bound labelled cortisol was determined. Cross-

reactions were calculated for each of the steroids and the results are shown in Table 5.1. These results were compared with those obtained by the producers of the antibody.

Steroid	Cross-reactivity for PAM 258 (%)	Cross-reactivity Guildhay results (%)
Corticosterone	1.2	18.6
Cortisone	1.1	<0.1
Prednisolone	12	31
17-hydroxyprogesterone	1.5	0.3
6-β-hydroxycortisol	3.2	

Table 5.1. *Results of cross-reactivity experiments with salivary cortisol assay, using PAM 258, comparing results obtained by Guildhay laboratories.*

5.2.10 Summary of Salivary Cortisol Assay Development

Salivary cortisol assay was developed initially using SAPU cortisol antibody. However, this was found to have a sensitivity of not less than 1.2nmol/L. This was felt to be inadequate for the purposes of this project in being able to detect low levels of cortisol in saliva. A more sensitive assay was developed with an antibody from Guildhay laboratories (PAM 258). Using various antibody dilutions and sample sizes, the optimum combination was found which gave good sensitivities and also good duplication of sample results. The final assay gave a sensitivity of 0.9 nmol/L, using an antibody dilution of 1 in 50 and sample sizes of 50 μl.

The coefficient of variation of the assay was 9% for intra-assay variation, and 12% for inter-assay variation for both high and low concentrations.

5.3 Salivary 17-Hydroxyprogesterone Assay Development

The salivary 17-OHP assay developed for this study was adapted from a previously developed capillary blood spot assay (Chearskull 1985). Using similar sample and standard volumes, incubation times and wash methods (as described earlier), and using PAM 259, the standard antibody used in the laboratory for routine serum and 17-OHP blood spot estimations, a standard curve was obtained which was adequate for use in salivary 17-OHP estimations. The assay sensitivity was 0.1 nmol/L.

5.4 Salivary Androstenedione Assay Development

There has been little published work to date on the measurement of androstenedione in saliva in normal children. Assay of androstenedione in serum

and blood spot samples has come across problems with the presence of steroid conjugates that interfered with the assay. The main decision with salivary androstenedione measurement was whether an extraction step was necessary.

5.4.1 Comparison of extraction assay and direct androstenedione assay for Salivary Androstenedione

Comparison of standard curves from extraction assay and direct assay, showed no significant difference in standard curves (Figs. 5.8; 5.9). There was a slight reduction in assay sensitivity between extraction and direct assay (44 pmol/l and 27 pmol/l). However, measurement of androstenedione in saliva samples resulted in much higher values being obtained by the extraction assay compared to the direct method, as has been described previously (Thomson 1991).

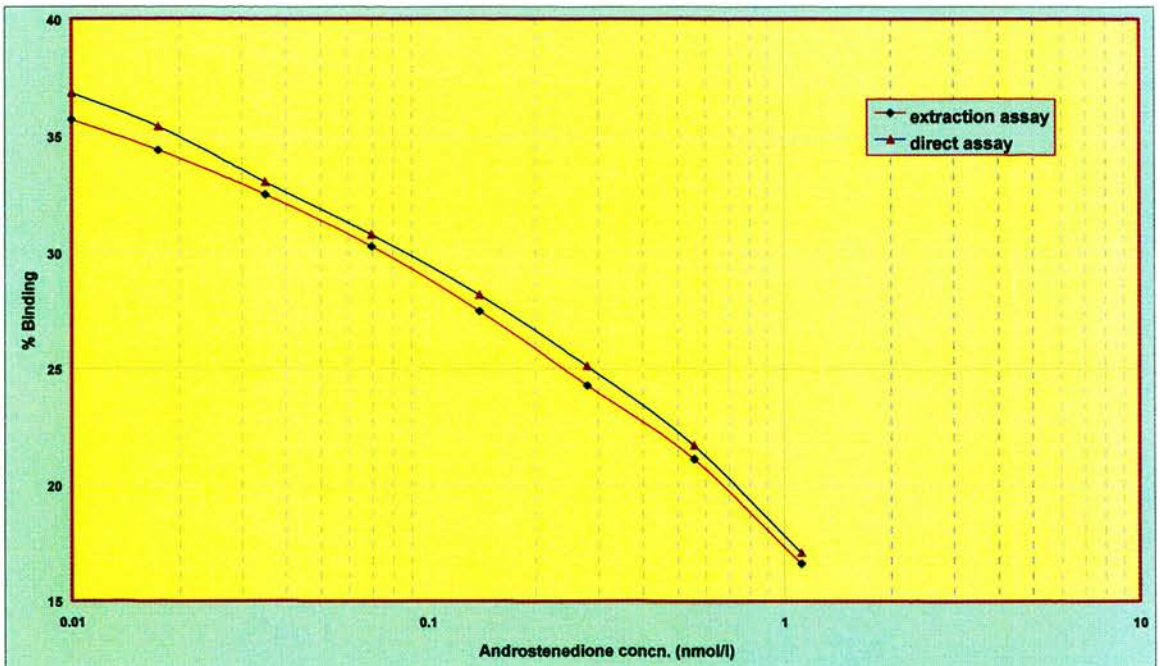

Fig. 5.8. Salivary androstenedione assay. Comparison of direct and extraction assays.

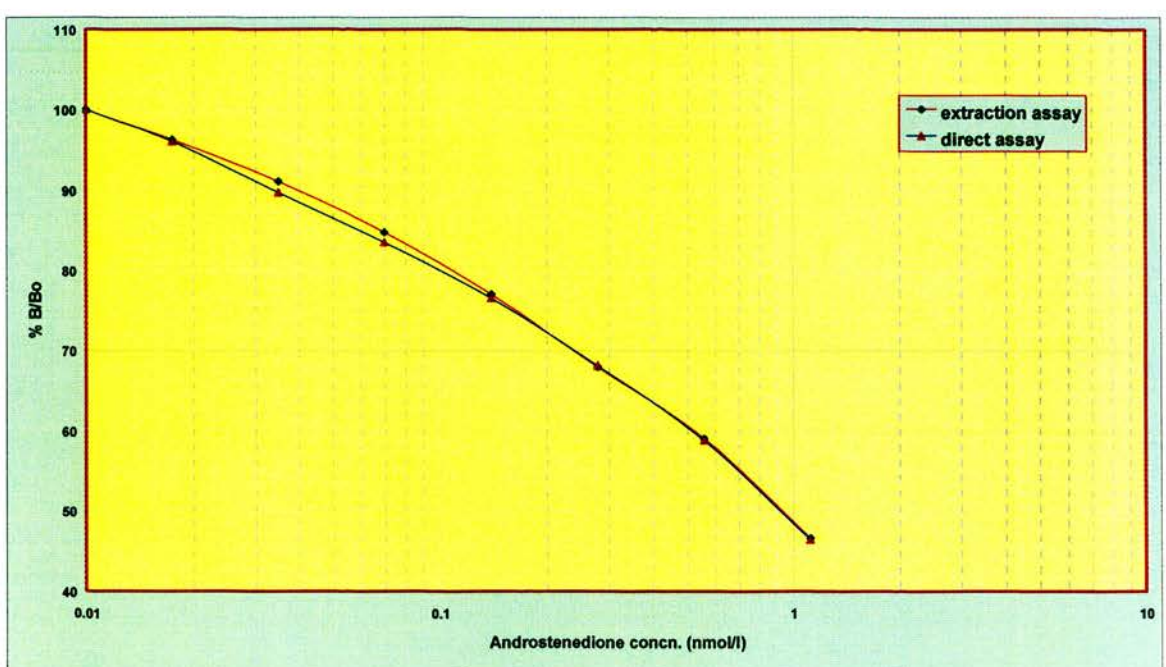

Fig. 5.9. Salivary androstenedione standard curves. Comparison of %B/Bo against androstenedione concentration for extraction and direct assays.

5.4.2 Comparison of standard curves for Salivary Androstenedione using different sample volumes

Comparing standard curves using 50 μl and 100 μl samples, it was noted that the sensitivity was reduced using the smaller volume size.

5.4.3 Antibody dilution curve for androstenedione assay

A modified Kelly curve was constructed using the same method as for the cortisol assay. From this, an antibody dilution of 1 in 200,000 was found to give optimal performance for the salivary androstenedione standard curve. Using this antibody dilution and extraction method, the final assay sensitivity was 35pmol/L.

5.5 Capillary Blood Spot Cortisol Assay Development

5.5.1 Introduction

Development of the capillary blood spot assay had several different problems to the salivary cortisol assay. One of the main problems was that of cortisol-protein binding, and more specifically of protein-binding inhibition. Once this was overcome, the next problem was optimising the assay for use on blood spot samples.
Much initial development work was performed with the salivary cortisol assay in relation to antibody selection.

5.5.2 Standard Curve for capillary cortisol using Different Concentrations of ANS

The standard curves were constructed using various concentrations of ANS (0 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 1 mg/ml, 5 mg/ml and 10 mg/ml).
This was used as a protein-binding inhibitor. ANS generally reduced the antibody binding and lowered the sensitivity to an unacceptable level. A concentration of 1 mg/ml of ANS gave the optimum results in terms of sensitivity.

5.5.3 Comparison of ANS with Citrate/phosphate buffer in capillary cortisol assay

Standard curves were plotted using RIA buffer with ANS and citrate/phosphate buffer (Figs. 5.10; 5.11). It was found that citrate/phosphate buffer interfered less with antibody binding, and also improved sensitivity of the assay.

From the results, it was deduced that the use of citrate/phosphate buffer was the optimal buffer for sensitivity. This was therefore used in further development of the capillary blood spot assay.

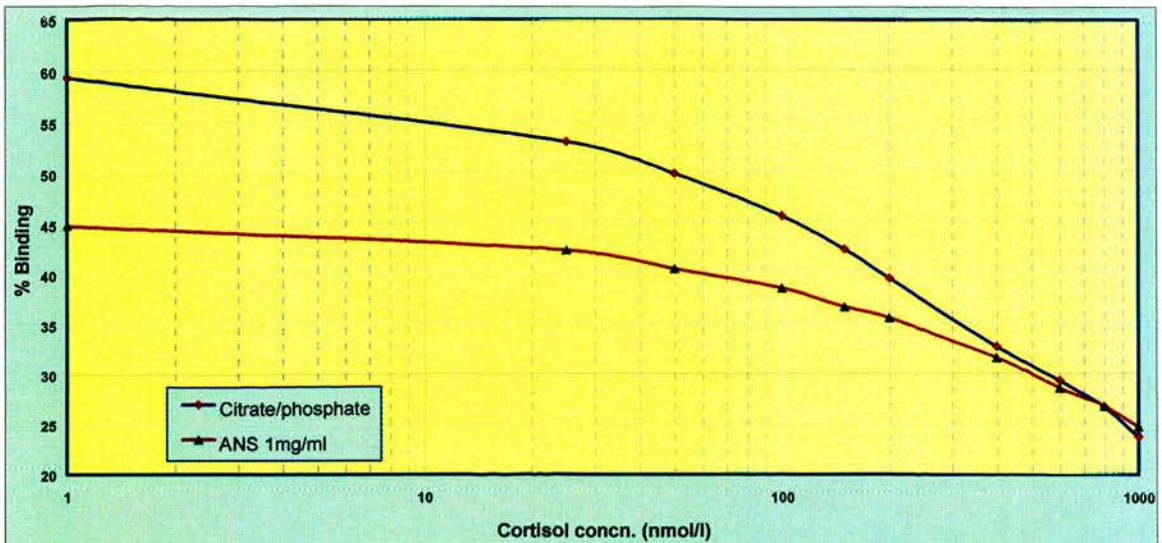

Fig. 5.10. Comparison of the effects of Citrate/phosphate Buffer and ANS on capillary cortisol standard curve - PAM 256.

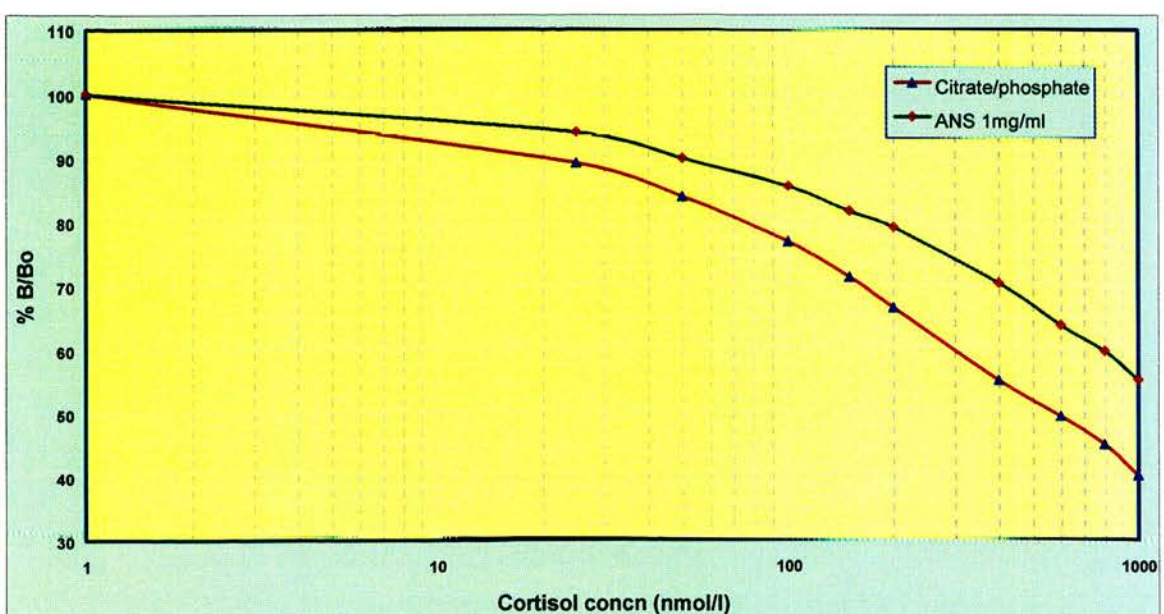

Fig. 5.11. Effects of Citrate/phosphate Buffer and ANS on Capillary Cortisol Standard Curve - %B/Bo against cortisol concn. for PAM 256

5.5.4 Comparison of different antibody dilutions, eluting fluid volumes and antibody preparations for capillary cortisol assay

A dilution of PAM 251 of 1 in 50 and 1 in 100 were compared. It was found that the standard curves were very similar, although there was slightly improved sensitivity when a dilution of 1 in 50 was used. When different volumes of eluting fluid were compared, the standard curve that employed 100 μl of eluting fluid gave the most sensitive standard curve. When comparing PAM 251, PAM 256, PAM 257 and PAM 258, the standard curves for each antibody preparation were very similar, with very little difference in sensitivities.

5.5.5 Comparison of standard curves at different temperatures and incubation times for capillary cortisol

The assay was repeated by initially eluting the samples overnight and then adding cortisol label and antibody the next day. This was then left to incubate for 1.5, and 3 hours at room temperature, and the results compared. The standard curve showed very poor sensitivity.

The assay was repeated, but the tubes were incubated at 37°C. The sensitivity did improve but still remained inadequate for the purposes of the study.

The assay was again repeated, but all reagents and eluting fluid were added to the tubes at the same time, the tubes were vortex-mixed, and left to incubate overnight at room temperature. The result of this was to increase antibody-cortisol binding and also to improve sensitivity to around 20 nmol/L whole blood.

5.5.6 Comparison of standard curves for capillary cortisol assay constructed from standard blood spot samples of different ages

Five standard curves were constructed using standard blood spot samples of varying ages. These were then compared (Figs. 5.12; 5.13). The standard curves were very similar, with very little difference in sensitivity, suggesting that the cortisol in the blood spot remains stable over time.

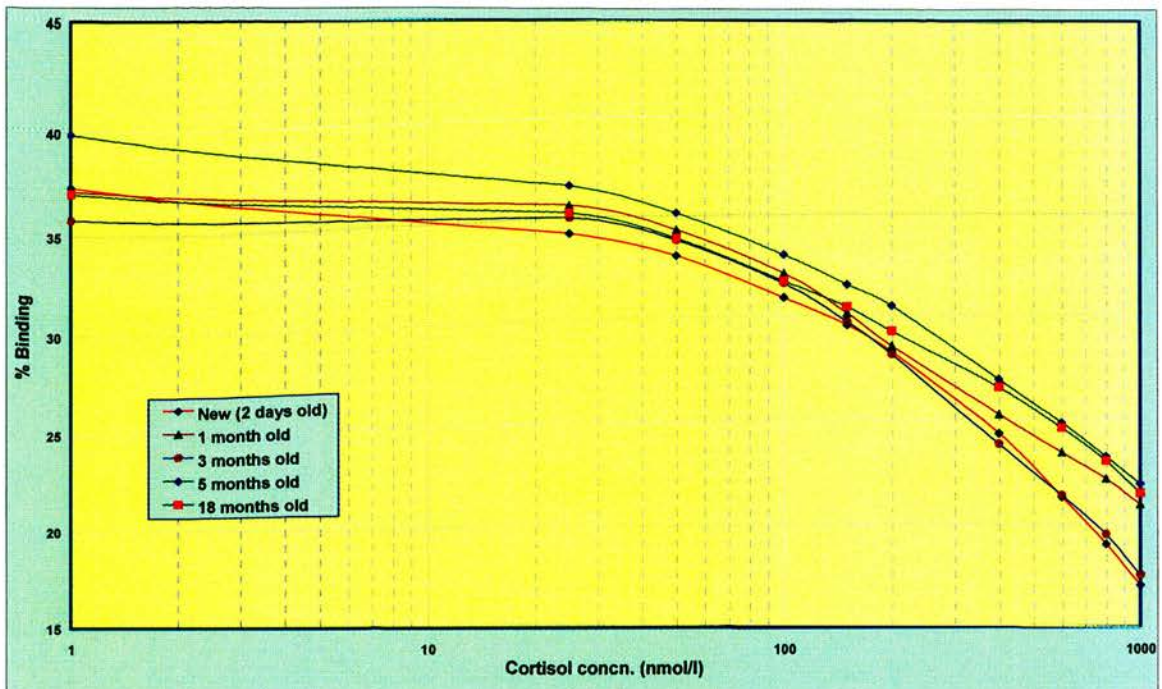

Fig. 5.12. Comparison of standard curves for capillary blood spot cortisol assay - Standard samples of increasing age

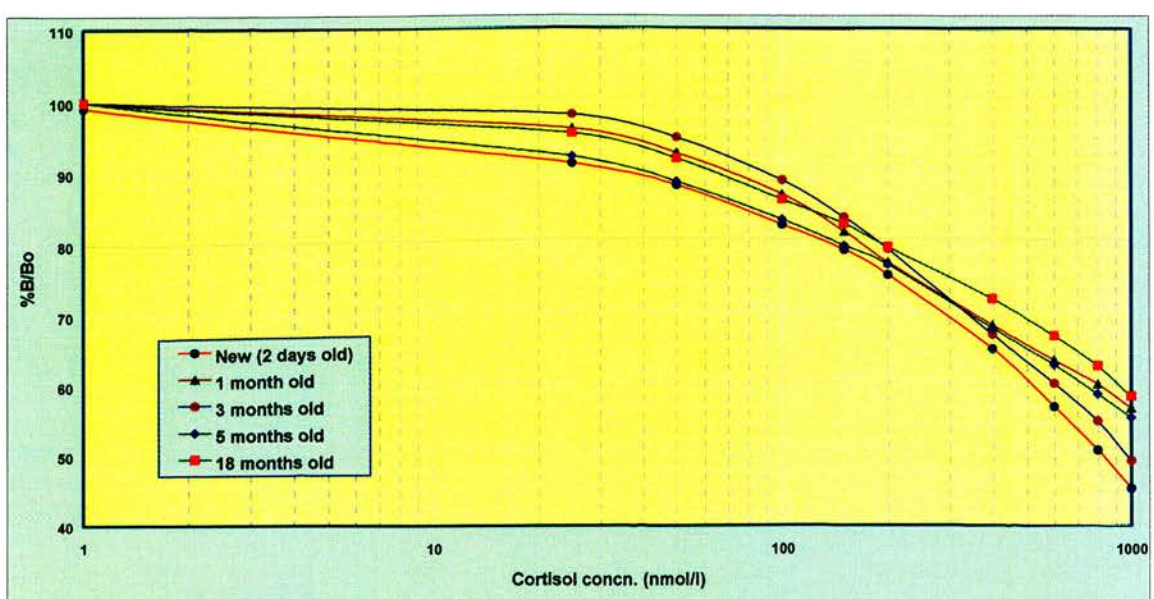

Fig. 5.13. Comparison of standard curves - Standard samples of increasing age, %B/Bo against cortisol concentration.

5.5.7 Summary of capillary blood spot cortisol assay development

The blood spot cortisol assay was developed from the salivary assay. There were more problems relating to antibody binding with this assay, as a consequence of the presence of cortisol binding globulin. The use of protein binding inhibitors to release cortisol from its binding protein also resulted in a significant reduction of antibody binding, particularly with ANS. This was partly resolved by the use of citrate/phosphate buffer at pH 4.0, and also by greatly prolonging incubation time by eluting with all the reagents overnight. The final assay had a sensitivity of 20 nmol/L whole blood (equivalent to 40 nmol/L serum).

6 Results

6.1 Control Population Data

Out of 340 schoolchildren whose parents gave consent, 172 (49%) subjects (91 girls, 77 boys) aged between 5 and 15 years provided up to six samples of saliva over a 24 hour period, at predetermined times (08:00, 11:00, 17:00, 20:00, 23:00, and 08:00 hours the following day). Of the 172 profiles, 21 (12.5%) were unsuitable for inclusion into the study because the samples were either too small in volume, or were blood-stained, or contained a significant amount of foreign material, e.g. food, on visual examination. A further four children were unable to provide adequate volumes of saliva. Therefore, 147 profiles (77 from girls, 70 from boys) were suitable for the study. The majority of the profiles were from primary schoolchildren (129) with only 18 samples from the secondary schoolchildren.

The median age of the boys in the control group at the time the profiles were taken was 8.9 years (range 5.0 to 13.6 years), and the median age for the girls was 10.0 years (range 5.3 to 14.4 years).

The heights and weights of the 77 female controls (Fig. 6.1, Fig. 6.2) and the 70 boys (Fig. 6.3, Fig. 6.4) who took part in the study were plotted against age and found to be comparable to the latest British height and weight reference charts. (Freeman et al 1995).

75 children (51%: 45 girls, 30 boys) provided 6 samples of saliva in 24 hours.

65 children (44%: 27 girls, 38 boys) provided 5 samples of saliva in 24 hours.

7 children (5%: 5 girls, 2 boys) provided up to 4 samples of saliva in 24 hours. Each sample of saliva was assayed for cortisol, 17-OHP, and androstenedione.

The maximum 8 a.m. concentrations, minimum concentrations, maximum/minimum ratio and area under the curve (AUC) for the 24 hour profiles were each plotted against age, body surface area, and body mass index for each steroid assayed.

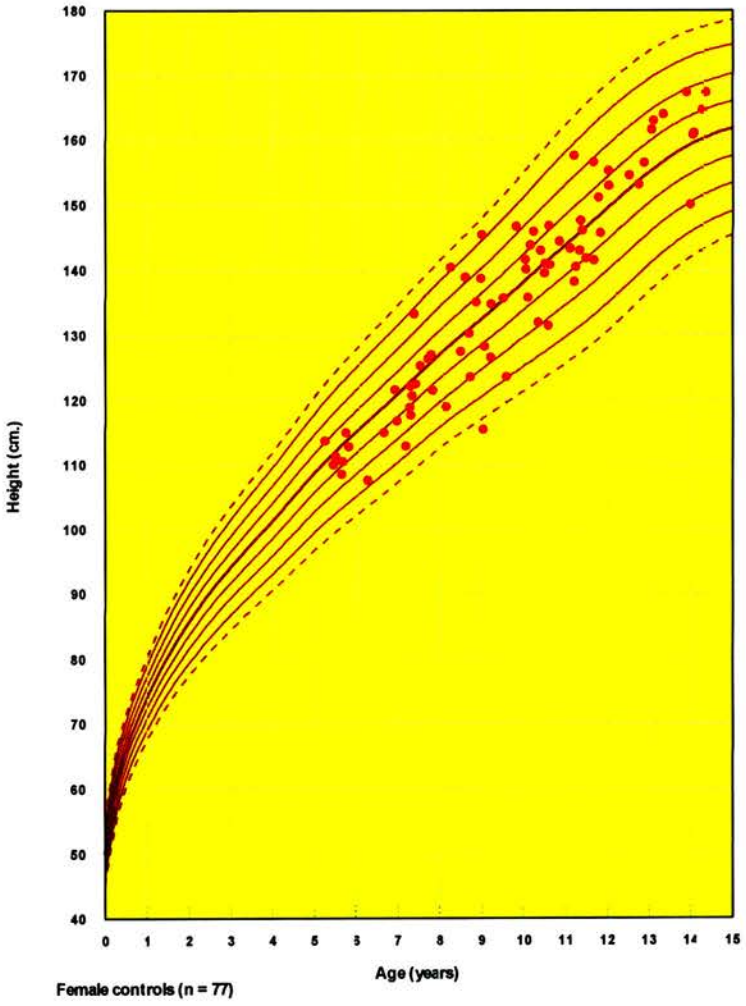

Fig. 6.1. Height against age for female control group on British standard height chart (1995 standards)

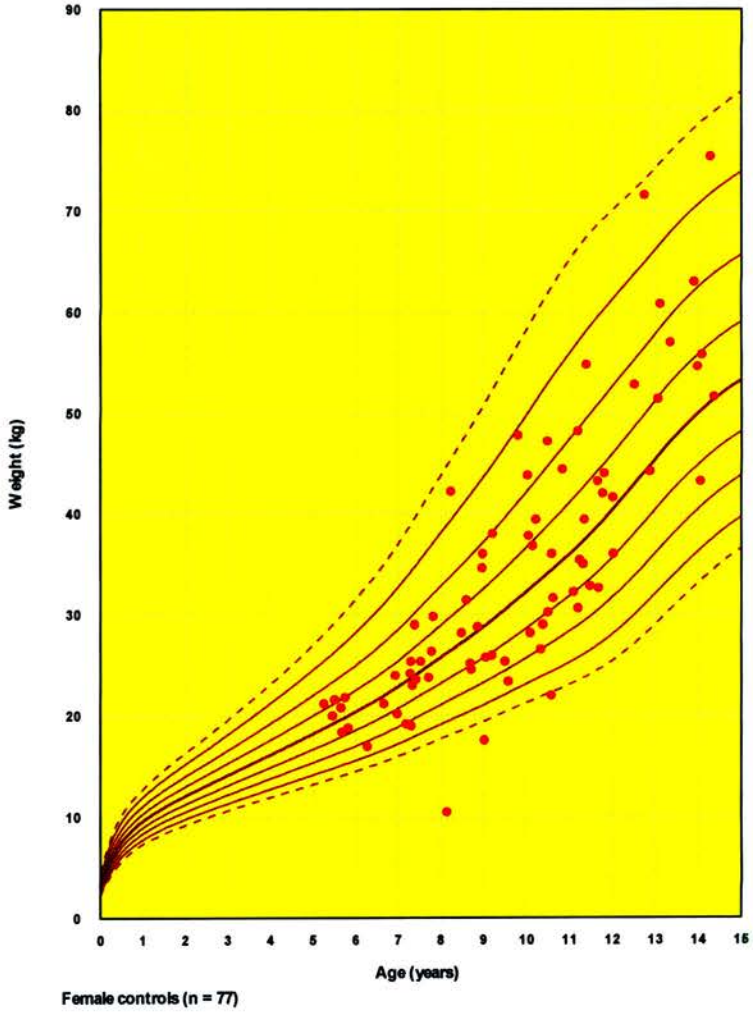

Fig. 6.2. Weight against age for female control group on British standa weight chart (1995 standards).

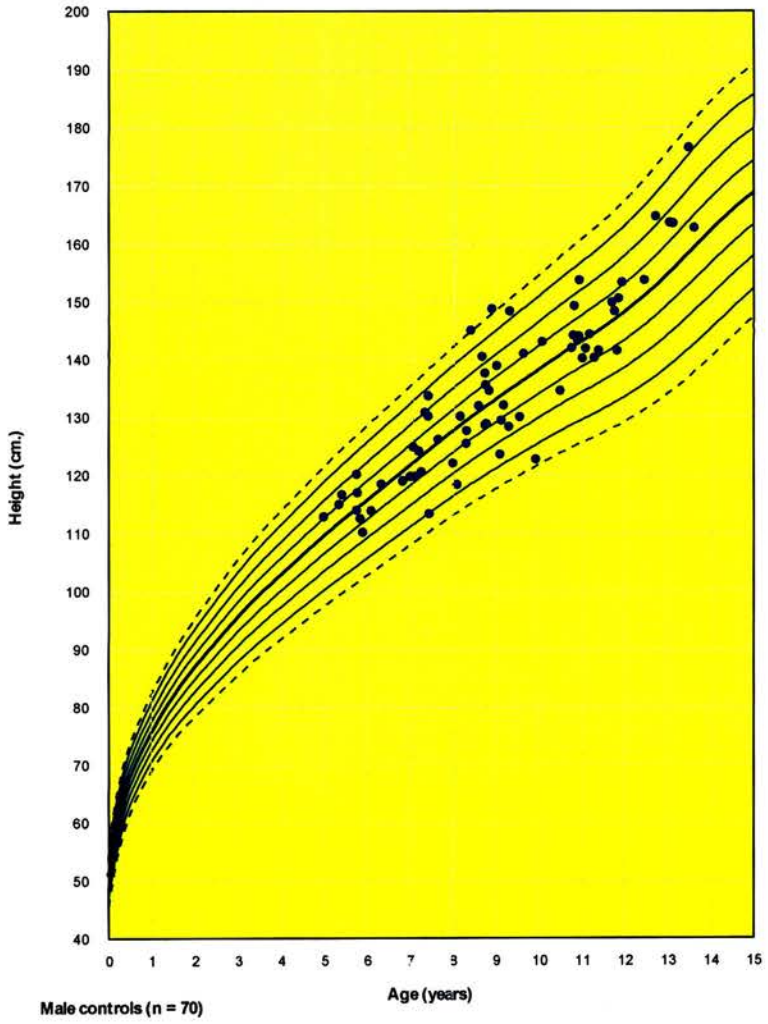

Fig. 6.3. Height against age for male control group on British standard height chart (1995 standards).

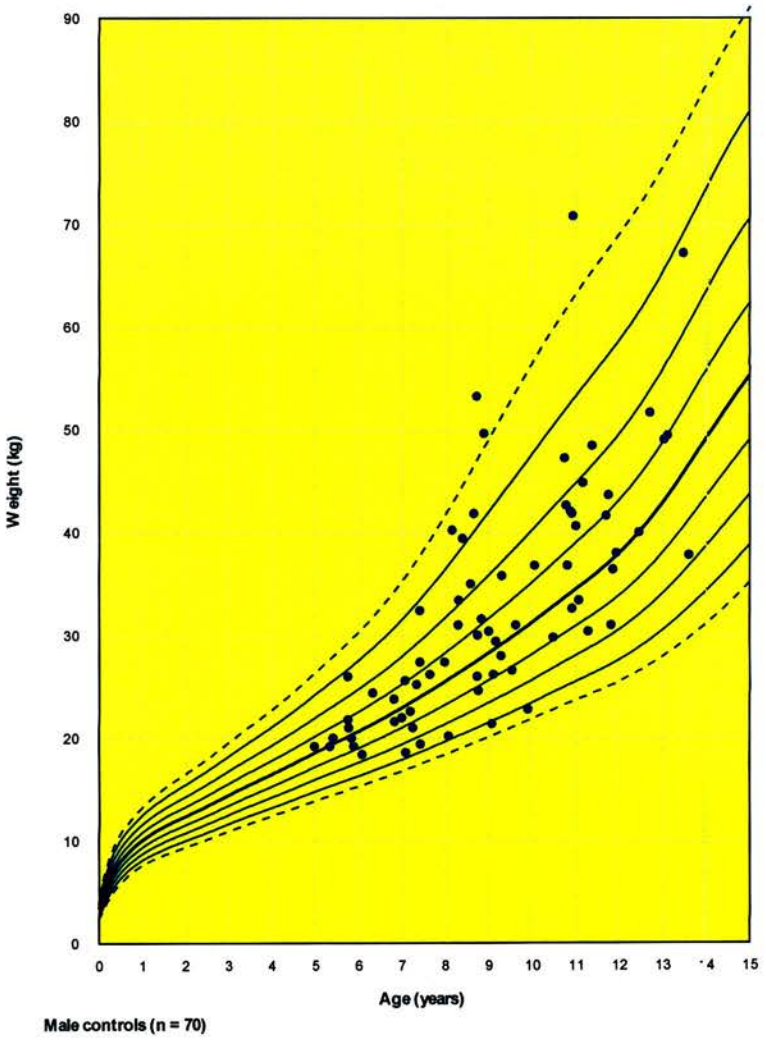

Fig. 6.4. Weight against age for male control group on British standard weight chart (1995 standards).

6.2 Patient Group Data

A total of 43 patients were suitable to be entered into the study. Thirty six patients provided samples of saliva. Fourteen patients (7 girls, 7 boys) provided salivary profiles when they were well and when they were unwell with an intercurrent illness. This was defined as a pyrexia of at least 38°C, and/or preventing them from going to school.

Thirty two patients (13 girls, 19 boys) also provided capillary blood spot samples at the same time as the salivary samples. Four of the patients preferred blood spot sampling to saliva collection, saying that collecting saliva was more difficult to perform, and took longer to collect.

Of these, 13 were patients with leukaemia (10 boys, 3 girls), and 20 were brain tumour survivors (8 boys, 12 girls). One boy had a B cell lymphoma, one boy a neuroblastoma and one boy a rhabdomyosarcoma of the jaw. All patients who took part were considered to be at risk of developing SAI owing to the dose of cranial irradiation they had received. A large proportion of these patients already had documented evidence of hypothalamo-pituitary dysfunction. Tables 6.1 and 6.2 show the different diagnostic groups of patients entered into the study together with details of the dose of cranial irradiation used and the calculated total dose to the hypothalamo-pituitary region that each child received.

Patient No.	Date Diagnosis	Date of Birth	Age at Diagnosis (years)	Diagnosis	Radiotherapy Dose	Pituitary Dose (cGy)
G01	07/03/1986	21/04/1981	4.9	ALL relapse	1800-Cr, 600-Cr	2400
G02	14/03/1989	14/05/1984	4.8	TBI - ALL relapse	1800,600-Cr 1440-TBI	1980
G03	15/11/1984	06/01/1982	2.9	TBI - ALL relapse	1800,540-Cr 1440-TBI	3800
G04	10/08/1989	16/02/1979	10.5	ependymoma	3600-Br 1980-Tum	5000
G05	16/08/1992	10/03/1980	12.4	germinoma	4000-Br	4000
G06	15/12/1980	05/10/1979	1.2	suprasellar asatrocytoma	2500	2500
G07	08/05/1990	16/07/1978	11.8	low grade astrocytoma	3000-Cr	3000
G08	28/07/1989	11/03/1987	2.4	Optic glioma	4500	4500
G09	29/07/1985	03/08/1981	4.0	optic glioma	5000-Br	5000
G10	30/01/1984	05/02/1980	4.0	optic glioma	5000-Br	5000
G11	22/08/1990	21/05/1979	11.3	medulloblastoma	3500-Br.Sp. 1980-PF	3500
G12	23/01/1992	24/06/1984	7.6	medulloblastoma	3500-Br. 3150-Sp. 1440-PF	3500
G13	29/03/1990	11/01/1980	10.2	medulloblastoma	3510-Br 3267-Sp 1503-PF	3510
G14	20/05/1991	24/11/1985	5.5	medulloblastoma	3500-Br.Sp. 1980-PF	3500
G15	10/07/1992	04/03/1984	8.4	medulloblastoma	3500-Br.Sp. 1980-PF	3500

Table 6.1. Female study patients in adrenal late effects study

Patient No.	Date Diagnosis	Date of Birth	Age at Diagnosis (years)	Diagnosis	Radiotherapy Dose (cGy)	Pituitary Dose (cGy)
B01	01/09/1981	07/05/1979	2.3	ALL	1800-Cr	1800
B02	12/03/1979	22/07/1974	4.6	ALL relapse	2400-Cr,1800-Test, 1800-Cr	3600
B03	12/11/1984	20/12/1982	1.9	ALL relapse	1800-Cr, 2400-Test	1800
B04	01/04/1988	12/07/1982	5.7	TBI - ALL relapse	600-Cr 1440-TBI	3800+1800
B05	19/09/1985	20/12/1980	4.7	TBI - ALL relapse	1800-Cr, 1440-TBI	1800+1440
B06	27/08/1987	25/05/1986	1.3	TBI - ALL relapse	1440-TBI	1440; 3240+1800
B07	20/01/1982	09/10/1974	7.3	TBI - ALL relapse	1800-Cr + 600-Cr, 1200-TBI	3600
B08	31/01/1984	15/02/1977	7.0	TBI - AML	1200-TBI	1200 (2Gy)
B09	27/10/1983	28/11/1981	1.9	TBI - AML	1200-TBI	1200 (2Gy)
B10	14/12/1987	27/08/1985	2.3	TBI - AMML	1440-TBI	1440
B11	23/05/1985	20/10/1981	3.6	TBI - Neuroblastoma	1200-TBI	1200 (2Gy)
B12	19/03/1986	09/07/1974	11.7	lymphoma (B cell NHL)	400-Test, 1440-TBI	1440
B13	24/06/1985	29/03/1980	5.2	Brain stem Glioma	5000	1500
B14	12/05/1989	29/07/1983	5.8	Glioma	4600	4600
B15	18/03/1993	17/01/1987	6.2	optic glioma	Surgery	
B16	10/10/1983	18/02/1977	6.6	optic glioma	4000	4000
B17	26/07/1991	03/10/1980	10.8	Germ cell Tumour Brain	3500-Br & Sp	3500
B18	16/10/1989	30/11/1978	10.9	medulloblastoma	3500-Br & Sp, 1980-PF	3500
B19	14/09/1987	18/09/1984	3.0	medulloblastoma	3500-Br & Sp, 1980-PF	3500
B20	03/03/1986	23/10/1984	1.4	medulloblastoma	4000-Br & Sp, 1980-PF	4000
B21	28/06/1985	23/06/1982	3.0	rhabdomyosarcoma of jaw	4500	3414

Table 6.2. Male study patients in adrenal late effects study

6.3 Salivary Steroid Profiles

6.3.1 Control Group

6.3.1.1 Salivary Cortisol

A diurnal variation of salivary cortisol was noted in all profiles (See Fig. 6.5). In all the profiles received, a peak salivary cortisol value was obtained in at least one of the 8 a.m. samples. The median morning salivary cortisol concentration was 14.5 nmol/L (reference range of 2.5% to 97.5%, 6.5 – 28.1 nmol/L). The median minimum salivary cortisol concentration (11 p.m.) was 1.4 nmol/L (reference range, 2.5% to 97.5%, <0.9 – 5.1 nmol/L) (See Fig. 6.6). Using the Mann-Whitney non-parametric test, no sex difference was demonstrated for salivary cortisol concentration.

There was no correlation between age and maximum salivary cortisol concentration ($r = -0.127$, $p = 0.176$), minimum cortisol concentration ($r = 0.044$, $p = 0.393$), maximum/minimum cortisol ratios ($r = 0.015$, $p = 0.278$), and AUC ($r = -0.048$, $p = 0.170$). There was also no correlation when similar curves were plotted against body surface area, and body mass index.

38 samples were found to have salivary cortisol concentrations below the limit of sensitivity of the assay (0.9nmol/L). These samples were taken either in the late afternoon or evening, whereas all morning samples had detectable cortisol concentrations.

In 138 of the profiles, two consecutive 8 a.m. samples were obtained. Comparing these two groups revealed that there were similar results in both groups. Median values were 12.2 and 10.3 mmol/L respectively, and the standard deviation was 5.8 and 6.1 respectively. The two groups also had a similar spread of results (minimum values were 1.0 and 1.3 mmol/L; maximum values were 30.4 and 35.9 mmol/L respectively). The correlation coefficient for the two groups was 0.16.

From the control data for salivary cortisol, a normal reference range was derived (Table 6.3).

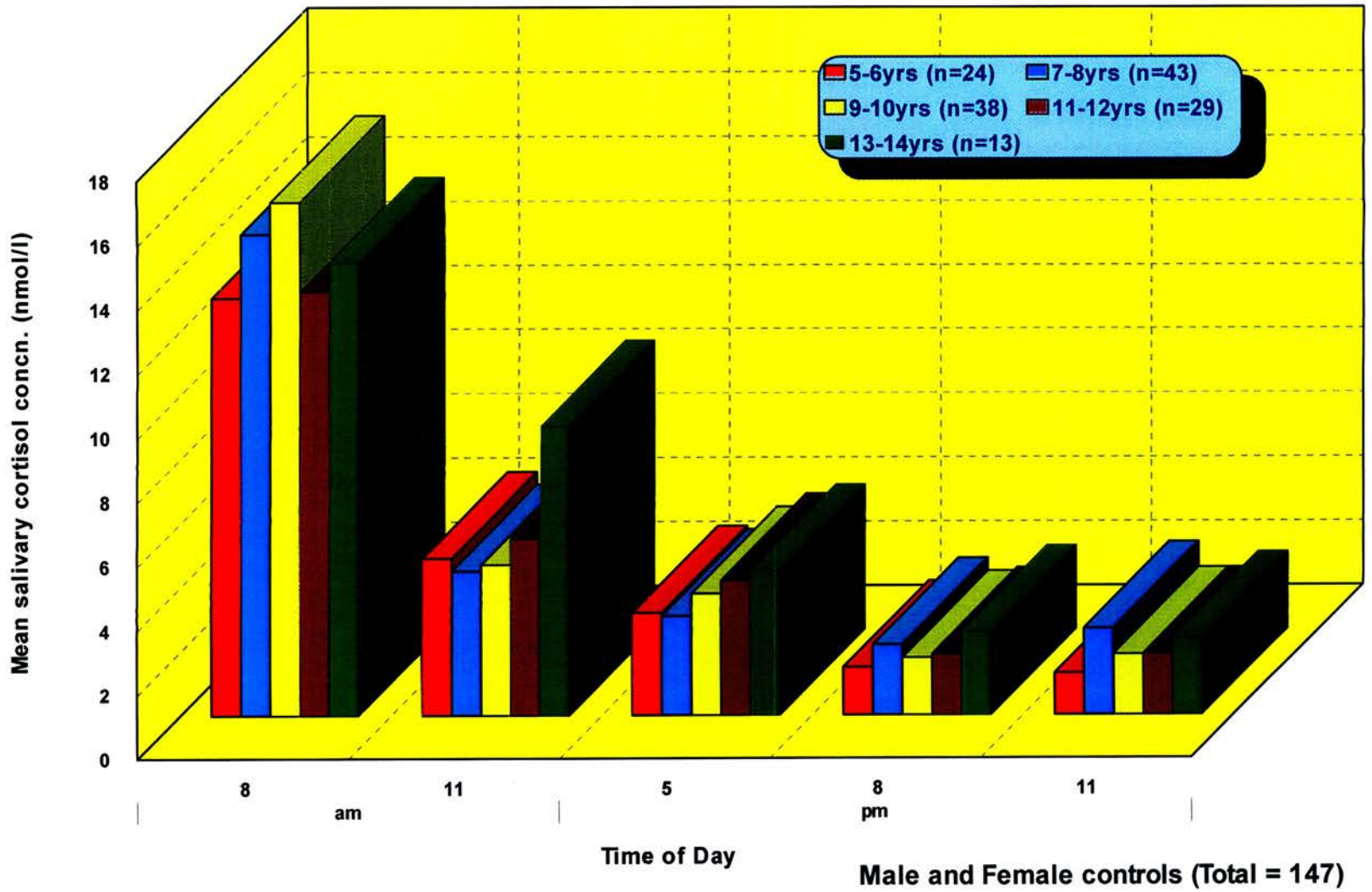

Fig. 6.5. Age distribution of median salivary cortisol profiles.

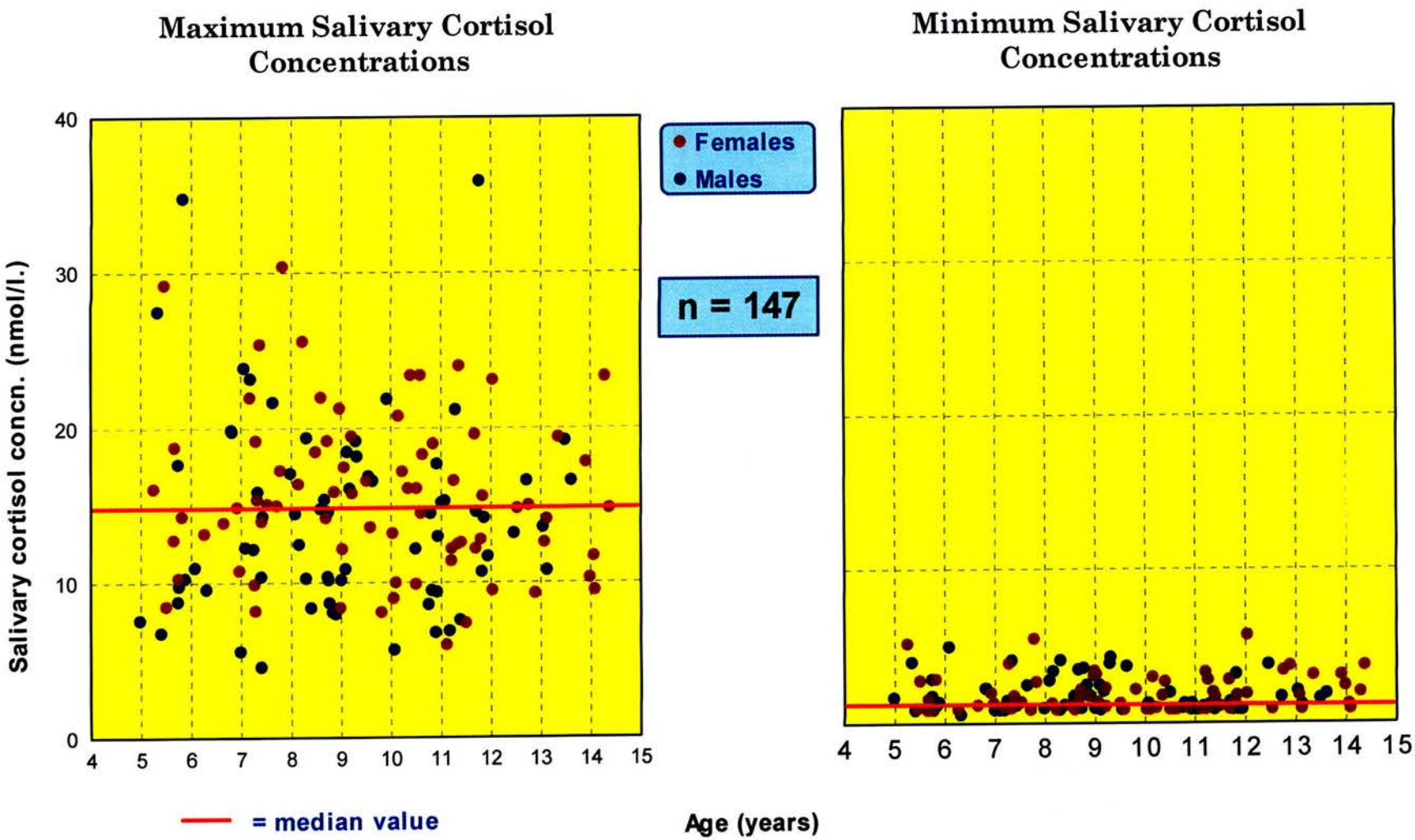

Fig. 6.6. Maximum and minimum salivary cortisol concentrations in the control population.

Morning (8a.m.)	
Median	14.5
Reference Range (2.5% - 97.5%)	6.5 - 28.1
Evening (8p.m.)	
Median	1.4
Reference Range (2.5% - 97.5%)	<0.9 - 5.1

Table 6.3. Salivary cortisol concentrations (in nmol/L) in healthy children aged 5 to 15 years (n=147))

6.3.1.2 Salivary 17-Hydroxyprogesterone

A diurnal variation was noted in the salivary 17-OHP profiles. (Figs. 6.7, 6.8). There was a small but significant difference in maximum salivary 17-OHP concentration between girls and boys in the control group ($p = 0.0142$). The median peak morning salivary 17-OHP concentration was 0.3 nmol/L for girls and 0.2 nmol/L for boys (Fig. 6.9). In girls, maximum salivary 17-OHP concentration rose significantly with age. However, this was only just within the level of significance ($r = 0.232$, $p = 0.044$; Fig. 6.10). No correlation was found between peak morning salivary 17-OHP and either body mass index ($r = 0.096$, $p = 0.411$), or body surface area ($r = 0.096$, $p = 0.058$).

In boys, no correlation was found between peak morning 17-OHP concentration and either age, body surface area ($r = 0.178$, $p = 0.159$) or body mass index ($r = 0.046$, $p = 0.717$). 17-OHP concentration did rise with age, but the correlation was only just out with the level of statistical significance ($r = 0.243$, $p = 0.053$) (Fig. 6.11). There was also a significant difference in maximum/minimum ratios between girls and boys ($p = 0.0074$). There was no sex difference for AUC for 17-OHP profiles ($p = 0.11$) nor for minimum salivary 17-OHP concentration ($p = 0.12$). Using the salivary 17-OHP data obtained from the control group, an age-related reference range was defined for males and females up to 15 years of age (Table 6.4).

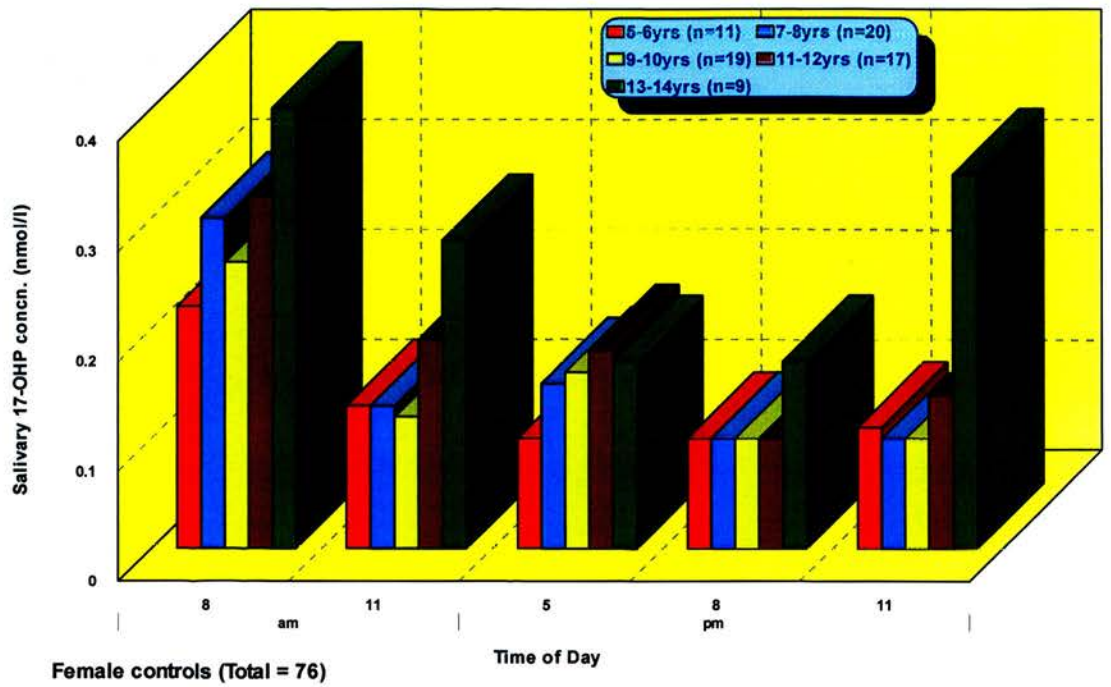

Fig. 6.7. Age distribution of median salivary 17-hydroxyprogesterone profiles in female controls

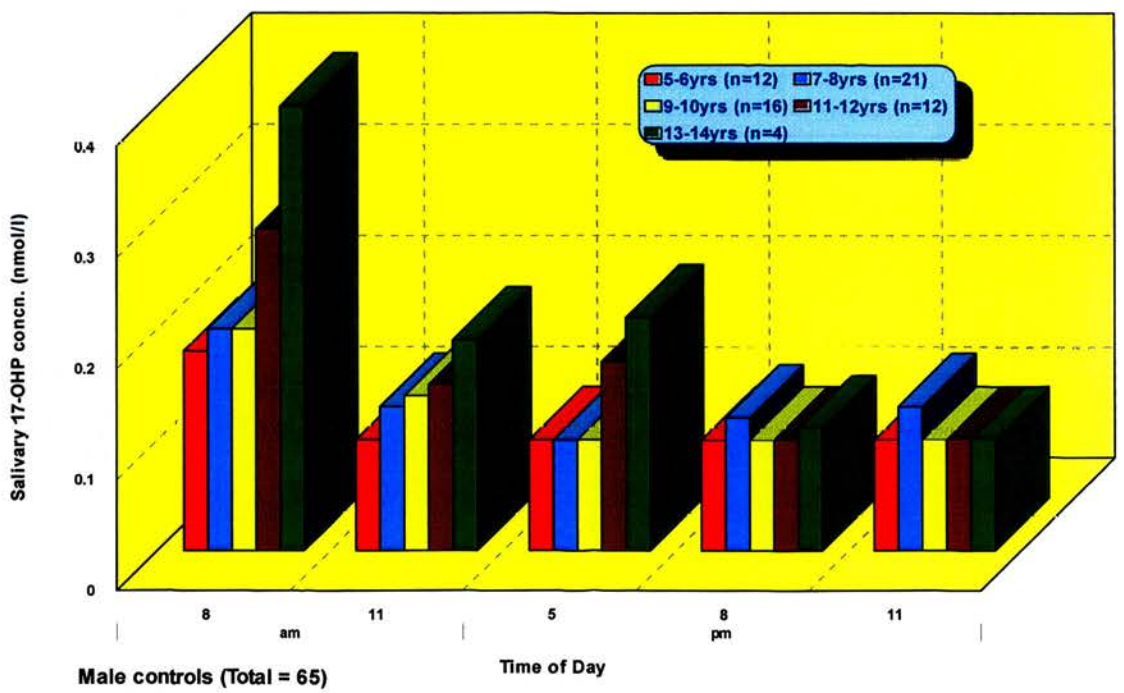

Fig. 6.8. Age distribution of median salivary 17-hydroxyprogesterone profiles in male controls.

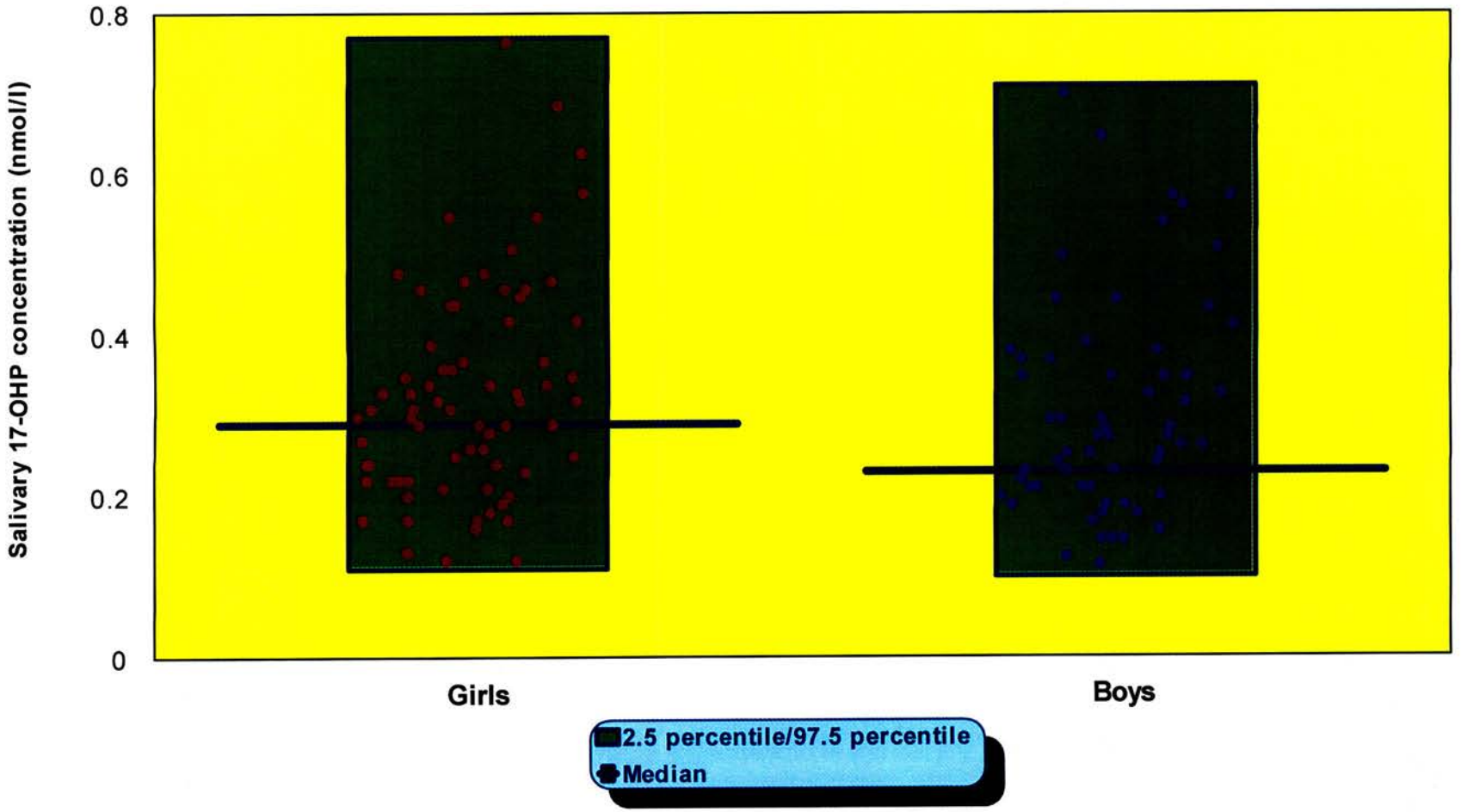

Fig. 6.9. Comparison of maximum salivary 17-hydroxyprogesterone concentrations between healthy boys and girls aged 5 to 15 years.

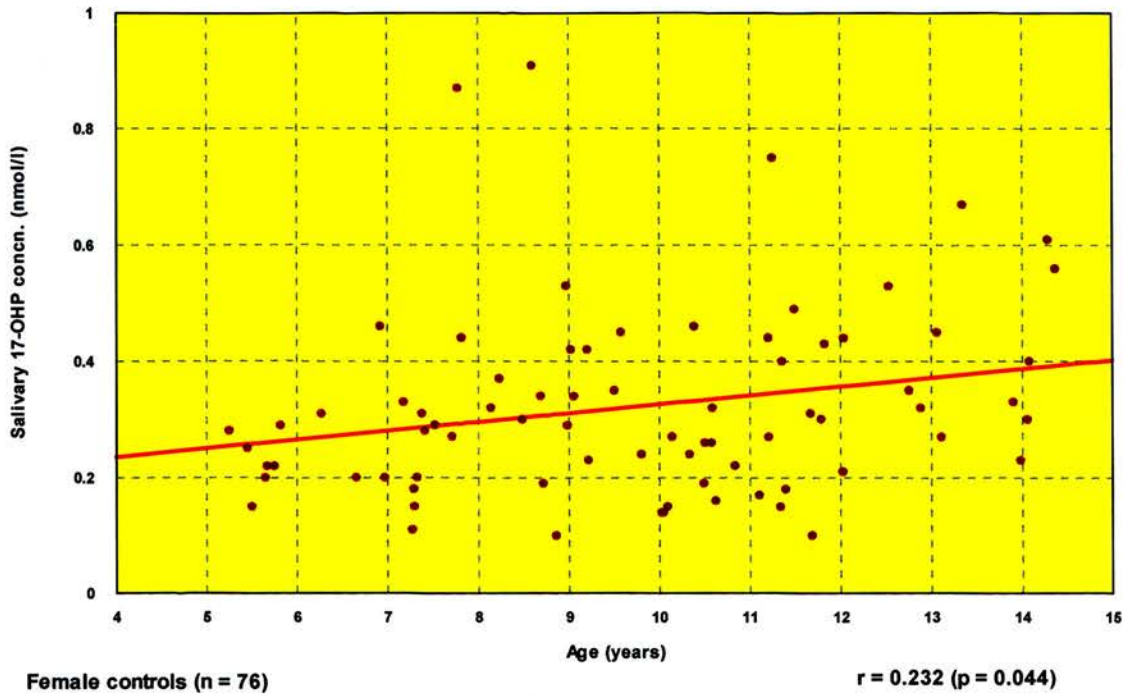

Fig. 6.10. Maximum salivary 17-hydroxyprogesterone levels against age in female controls.

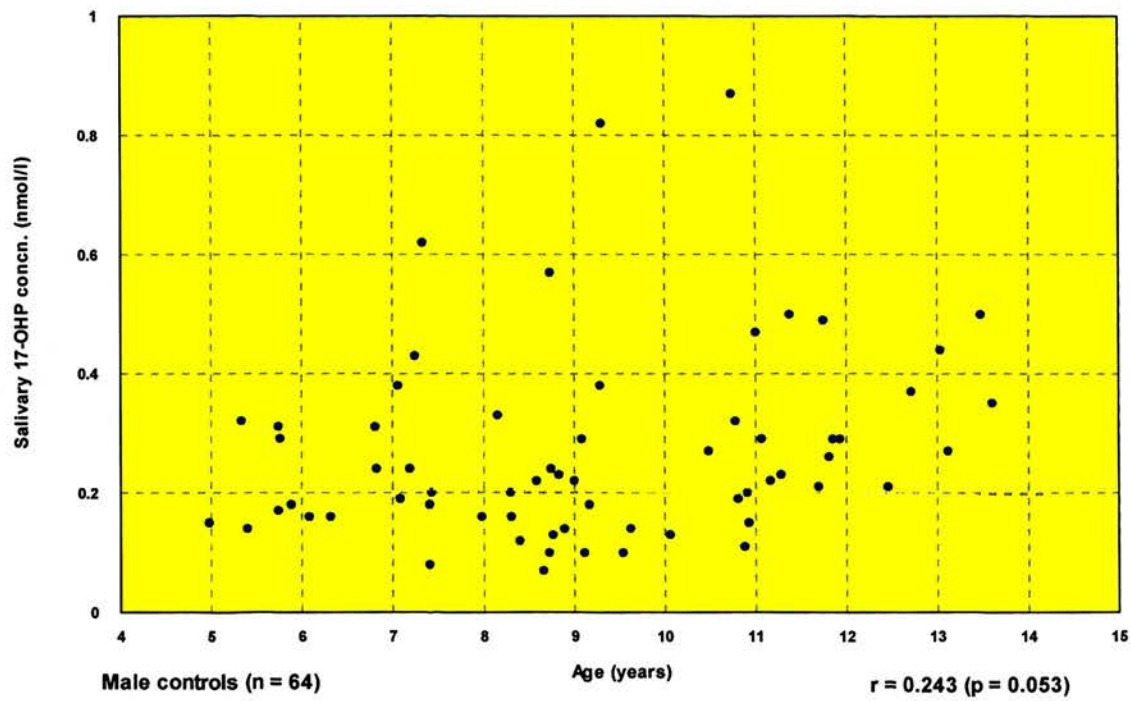

Fig. 6.11. Maximum salivary 17-hydroxyprogesterone concentration against age in male controls.

Girls		Morning (8a.m.)		Evening (8p.m.)	
Age (years)	n	Reference Range (2.5% - 97.5%)	Median	Reference Range (2.5% - 97.5%)	Median
5 to 6	11	0.2 - 0.4	0.2	<0.1 - 0.2	<0.1
7 to 8	20	0.1 - 0.9	0.3	<0.1	<0.1
9 to 10	19	0.1 - 0.5	0.23	<0.1 - 0.2	<0.1
11 to 12	17	0.1 - 0.7	0.3	<0.1 - 0.2	<0.1
13 to 15	9	0.2 - 0.7	0.4	<0.1 - 0.3	0.1
Boys		Morning (8a.m.)		Evening (8p.m.)	
Age (years)	n	Reference Range (2.5% - 97.5%)	Median	Reference Range (2.5% - 97.5%)	Median
5 to 6	12	0.1 - 0.3	0.2	<0.1 - 0.2	<0.1
7 to 8	21	<0.1 - 0.6	0.2	<0.1 - 0.2	<0.1
9 to 10	16	0.1 - 0.8	0.2	<0.1 - 0.3	<0.1
11 to 12	12	0.2 - 0.5	0.3	<0.1 - 0.2	<0.1
13 to 15	4	0.3 - 0.5	0.4	<0.1 - 0.2	0.1

Table 6.4. Reference range for salivary 17-OHP concentrations (in nmol/L) in healthy schoolchildren aged 5 to 15 years

Minimum salivary 17-OHP concentration was below the limit of sensitivity of the assay (0.1nmol/L) in 55 out of 76 girls, and 52 out of 65 boys.

In girls, the peak morning salivary 17-OHP concentration correlated well with age ($r = 0.367$, $p = 0.001$) and body surface area ($r = 0.283$, $p = 0.013$), but not with body mass index ($r = 0.106$, $p = 0.363$). Stepwise regression analysis showed that age was the most important predictor.

In boys, no such correlation was found.

In girls a significant correlation was found between AUC for salivary 17-OHP profiles and age ($r = 0.267$, $p = 0.026$) and body surface area ($r = 0.244$, $p = 0.042$) but not body mass index ($r = 0.136$, $p = 0.261$).

In boys no such correlation was found.

6.3.1.3 Salivary Androstenedione

A diurnal variation was also noted in the salivary androstenedione profiles (Figs. 6.12; 6.13), and values were also found to rise with age. There was a significant difference between female and male maximum salivary androstenedione concentrations ($p = 0.0084$). The median maximum concentrations were 0.4nmol/L for girls and 0.3nmol/L for boys (Fig. 6.14). In girls there was a significant relationship between maximum salivary androstenedione concentration and age, body surface area and body mass index ($r = 0.571$, $p<0.001$; $r = 0.552$, $p<0.001$; $r = 0.385$, $p=0.001$, respectively). Using stepwise regression analysis, the main predictor was found to be age (Fig. 6.15).

In boys, no such relationship was found (Fig. 6.16).

There was also a significant difference in AUC for boys and girls ($p = 0.0124$). The median value for girls was 4.7, for boys it was 3.3. There was no significant difference between girls and boys for maximum/minimum salivary androstenedione ratios and minimum salivary androstenedione concentrations. In girls, there was also a significant correlation between minimum salivary androstenedione concentration and age ($r = 0.548$, $p<0.001$), body surface area ($r = 0.586$, $p<0.001$), and body mass index ($r = 0.463$, $p<0.001$). Using stepwise regression analysis, the main predictor was found to be body surface area. This correlation was not noted in boys (age: $r = 0.113$, $p = 0.353$; body surface area: $r = 0.074$, $p = 0.550$; body mass index: $r = 0.081$, $p = 0.511$).

There was a significant correlation between AUC for salivary androstenedione profiles and age ($r = 0.575$, $p<0.001$), body surface area ($r = 0.580$, $p<0.001$), and body mass index ($r = 0.455$, $p<0.001$). Using stepwise regression analysis, the main predictor was found to be body surface area.

In boys, no correlation was found between AUC and either age ($r = 0.185$, $p = 0.144$), body surface area ($r = 0.062$, $p = 0.629$), or body mass index ($r = 0.002$, $p = 0.988$).

Using the salivary 17-OHP data obtained from the control group, an age-related reference range was devised for males and females up to 15 years of age (Table 6.4).

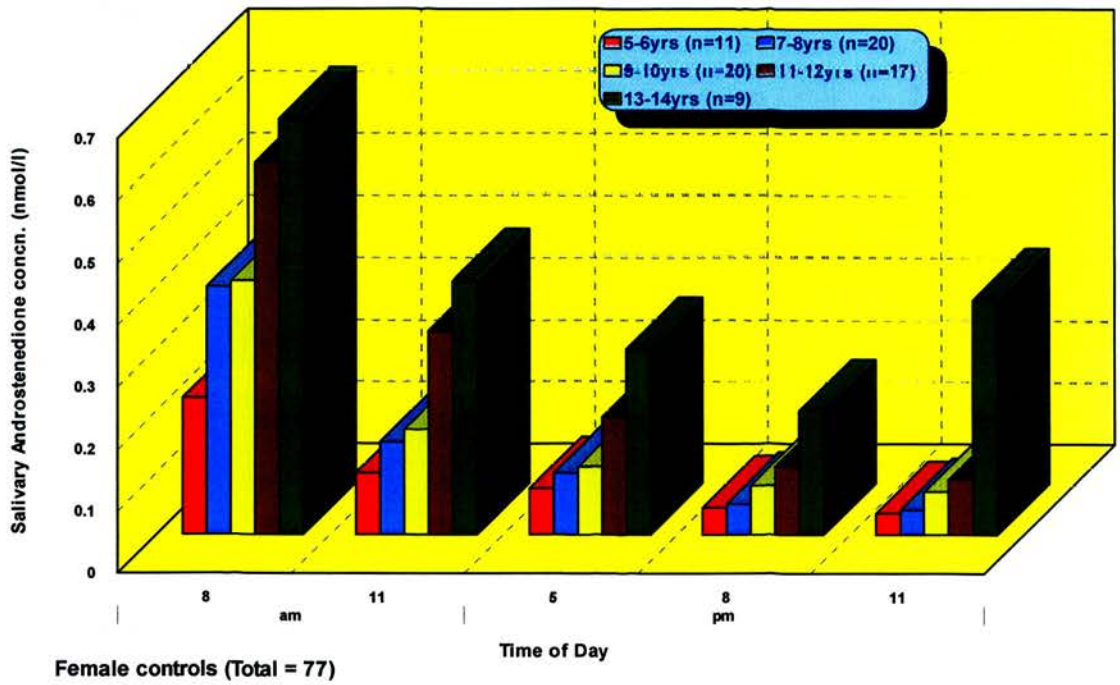

Fig. 6.12. Age distribution of median salivary androstenedione profiles in female controls.

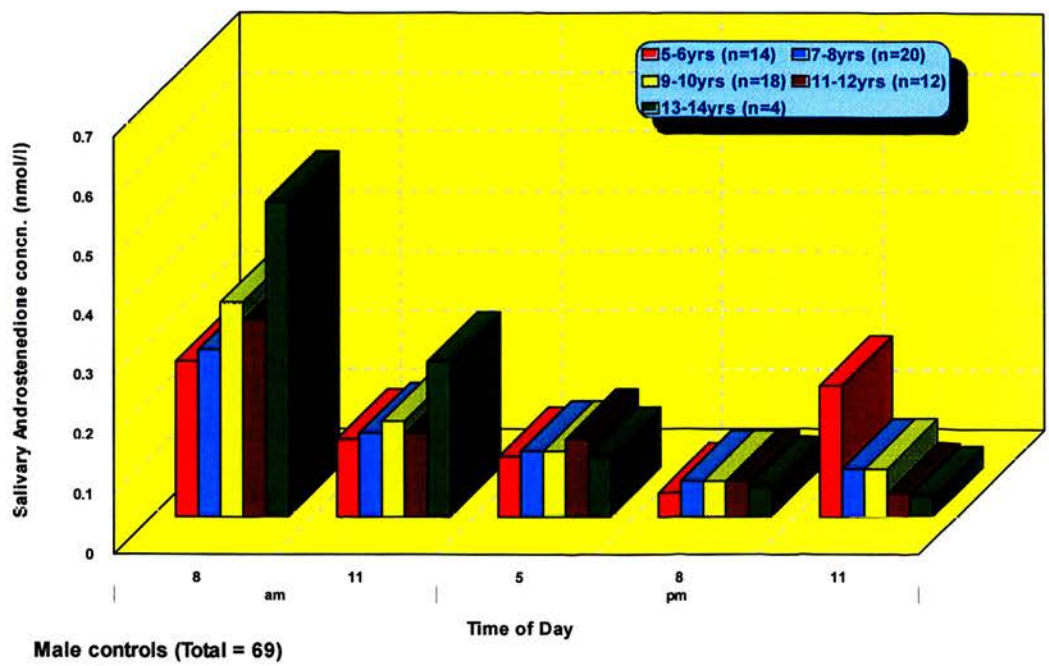

Fig. 6.13. Age distribution of median salivary androstenedione profiles in male controls.

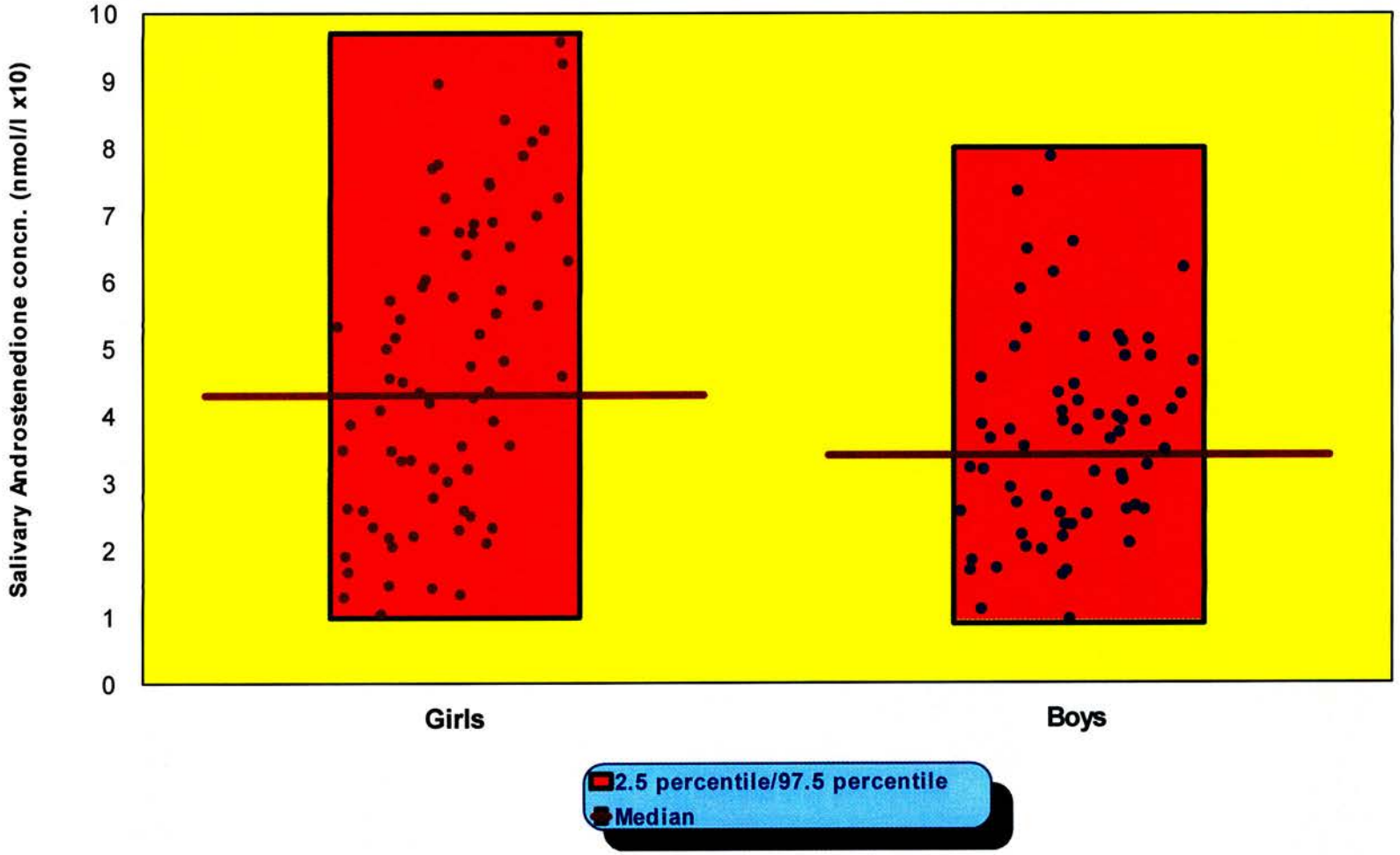

Fig. 6.14. Comparison of maximum salivary androstenedione in healthy girls and boys aged 5 to 15 years.

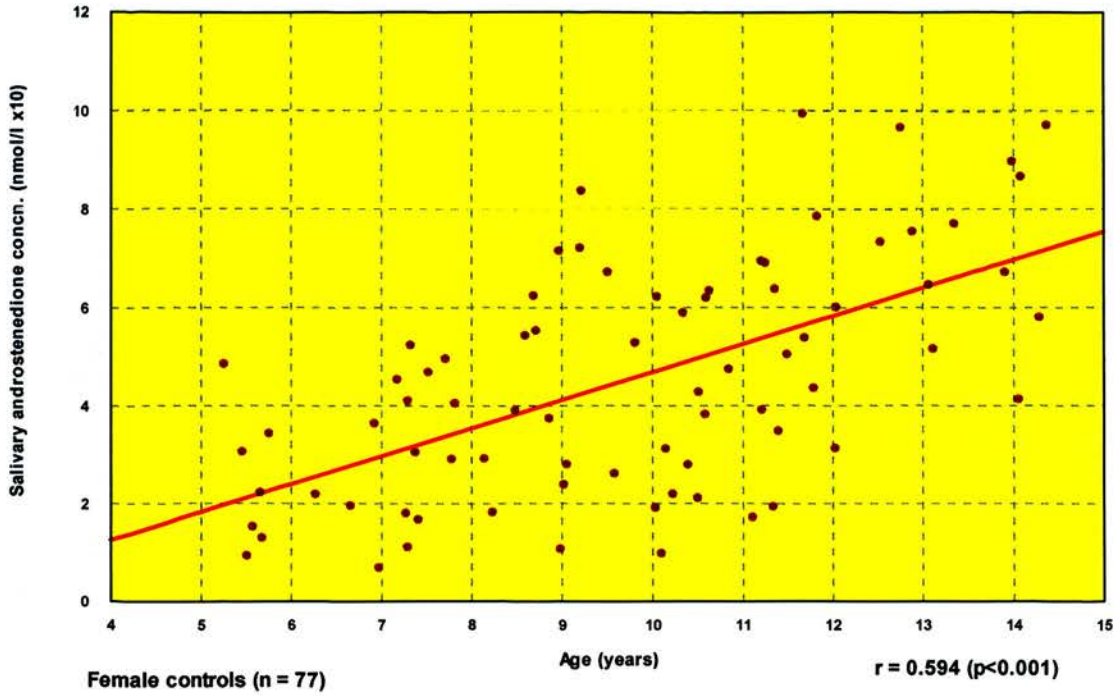

Fig. 6.15. Maximum salivary androstenedione concentration against age in female controls

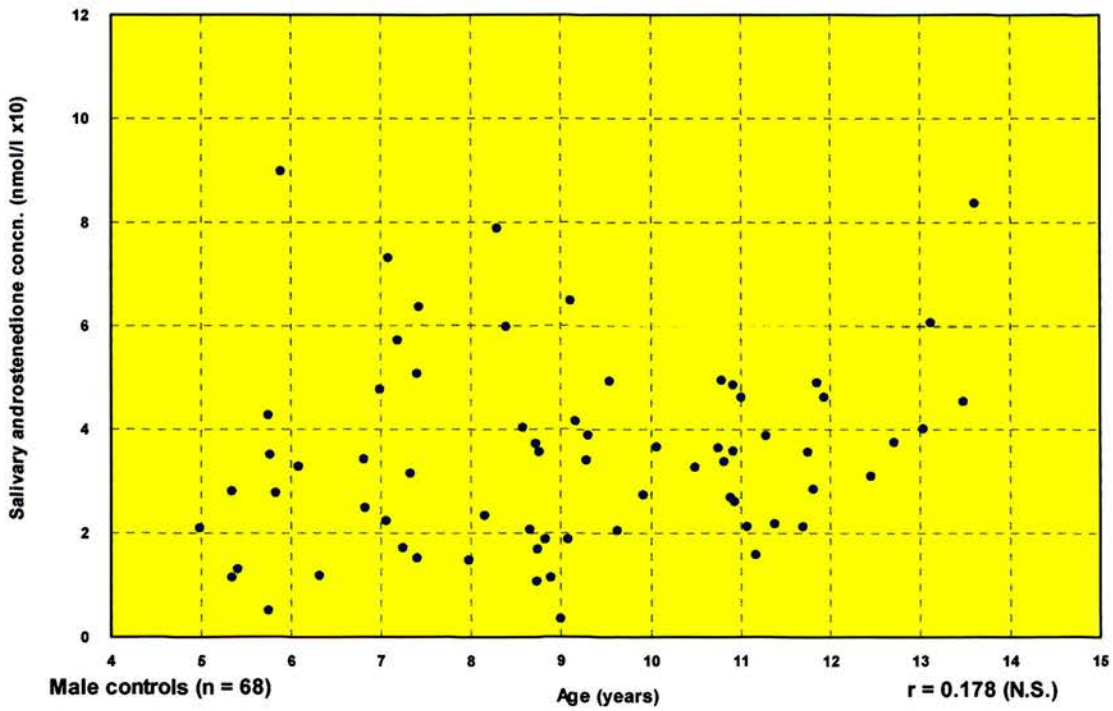

Fig. 6.16. Maximum salivary androstenedione concentration against age in male controls

Girls		Morning (8a.m.)		Evening (8p.m.)	
Age (years)	n	Reference Range (2.5% - 97.5%)	Median	Reference Range (2.5% - 97.5%)	Median
5 to 6	11	0.1 - 0.5	0.20	<0.04 - 0.1	0.04
7 to 8	20	0.1 - 0.7	0.40	<0.04 - 0.1	0.04
9 to 10	20	0.1 - 0.8	0.40	<0.04 - 0.2	0.05
11 to 12	17	0.2 - 1.0	0.60	<0.04 - 0.4	0.08
13 to 15	9	0.04 - 1.0	0.70	0.10 - 0.4	0.20

Boys		Morning (8a.m.)		Evening (8p.m.)	
Age (years)	n	Reference Range (2.5% - 97.5%)	Median	Reference Range (2.5% - 97.5%)	Median
5 to 6	14	0.07 - 0.8	0.30	<0.04 - 0.07	0.04
7 to 8	20	0.07 - 0.8	0.30	<0.04 - 0.2	0.06
9 to 10	18	0.20 - 0.6	0.40	<0.04 - 0.15	0.04
11 to 12	12	0.2 - 0.5	0.30	<0.04 - 0.14	0.04
13 to 15	4	0.3 - 0.8	0.50	<0.04 - 0.12	0.05

Table 6.5. Reference range for salivary androstenedione concentrations (in nmol/L) in healthy schoolchildren aged 5 to 15 years

6.3.2 Salivary Steroid Ratios

Ratios of cortisol to 17-OHP, cortisol to androstenedione and 17-OHP to androstenedione, were calculated for each maximum and minimum salivary cortisol concentration for each control subject.

The aim was to determine the relative change in salivary steroid levels at peak and trough cortisol concentrations, to assess whether this may be of value in detecting hypoadrenalism. We hypothesised that in SAI, 17-OHP is "shunted" down the cortisol pathway to maximise cortisol output at the expense of androstenedione production, so the cortisol/17-OHP or cortisol/androstenedione ratios will rise.

The results were then plotted against age, body surface area and body mass index. For minimum concentrations that were below the limit of sensitivity of the assays the concentration was taken as that limit (i.e. 0.9nmol/L for salivary cortisol).

6.3.2.1.1 Salivary Cortisol/17-Hydroxyprogesterone Ratios

In girls, there was no significant correlation between the cortisol/17-OHP ratios at maximum salivary cortisol concentration and any of the parameters above (age: $r = -0.178$, $p = 0.126$, figure 6.17; body surface area: $r = -0.221$, $p = 0.056$; body mass index: $r = -0.202$, $p = 0.082$). The median value at maximum salivary cortisol concentration in girls was 55.6 (range 19.9 - 397.5).

In boys, there was a significant but weak negative correlation between the ratios at maximum salivary cortisol concentration and body surface area ($r = -0.25$, $p= 0.042$; figure 6.18). The ratios decreased with increasing body size. The median value at maximum salivary cortisol concentration in boys was 57.5 (range 9.9 - 273.8). Otherwise there was no significant correlation found between age ($r = -0.133$, $p = 0.285$) and body mass index ($r = -0.202$, $p = 0.102$).

For ratios at minimum salivary cortisol concentration, there was no correlation found for either boys and girls between the ratios at minimum salivary cortisol

concentration and age (girls: r = -0.008, p =0.943; boys: r =-0.011, p = 0.932), body surface area (girls: r = 0.089, p = 0.448; boys: r = -0.183, p = 0.137), and body mass index (girls: r = 0.081, p = 0.489; boys: r = -0.146, p = 0.238).

The median value at minimum salivary cortisol concentration in girls was 12 (range 3.2 to 57). In boys the median value was also 12 (range 3.9 to 154).

Using the Mann-Whitney test, there was no sex difference between the cortisol/17-OHP ratios at maximum and minimum salivary cortisol concentrations.

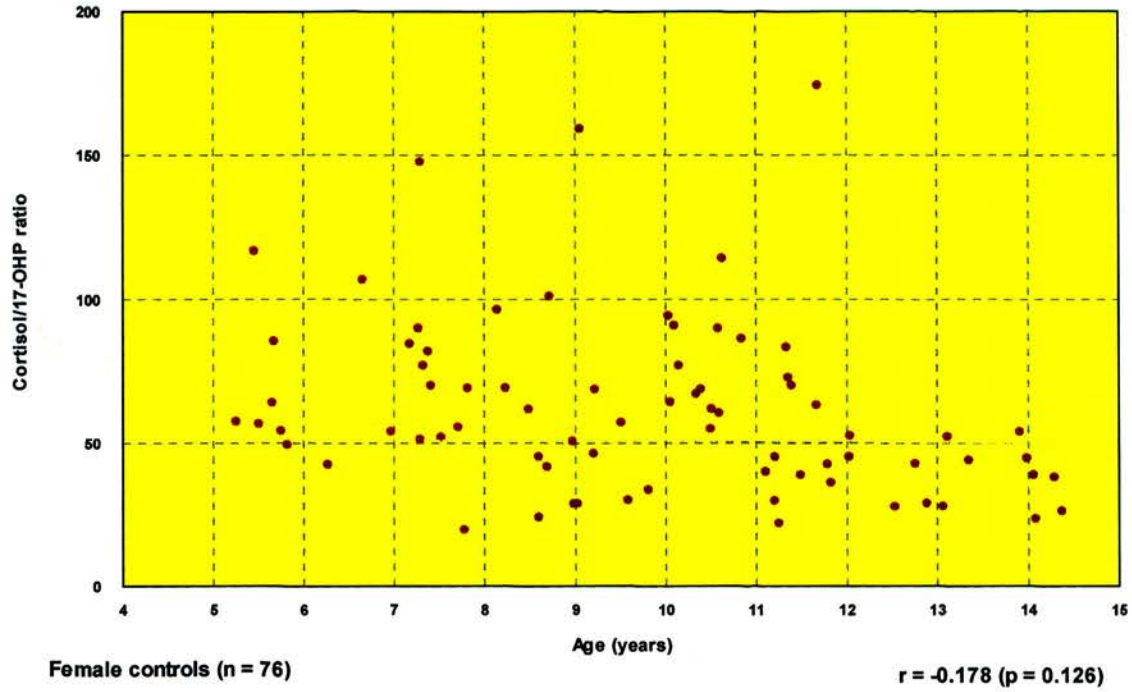

Fig. 6.17. Cortisol/17-hydroxyprogesterone ratio against age at maximum salivary cortisol concentration - female controls

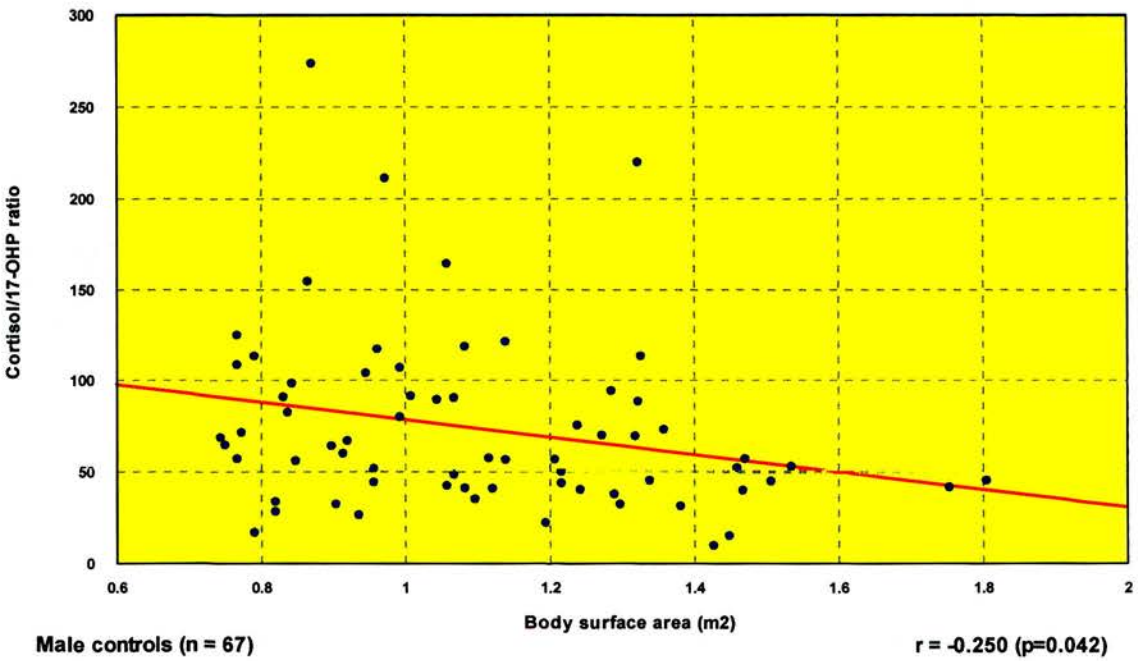

Fig. 6.18. Cortisol/17-hydroxyprogesterone ratio against body surface area for maximum salivary cortisol concentration - male controls

Salivary Cortisol/Androstenedione Ratios

In girls, there was significant negative correlation between salivary cortisol/androstenedione ratios at maximum salivary cortisol concentration and age ($r = -0.539$, $p<0.001$, figure 6.19), body surface area ($r = -0.423$, $p<0.001$), and body mass index ($r = -0.254$, $p = 0.026$). Using stepwise regression analysis, age was found to be the main predictor.

The median value at maximum salivary cortisol concentration in girls was 3.8 (range 1.2 to 15.7).

In girls there was also significant negative correlation between salivary cortisol/androstenedione ratios at minimum salivary cortisol concentration and age ($r = -0.270$, $p = 0.018$, figure 6.20), but not body surface area ($r = -0.184$, $p = 0.111$), nor body mass index ($r = -0.095$, $p = 0.414$).

The median value at minimum salivary cortisol concentration in girls was 2.2 (range 0 to 18).

In boys, there was no correlation found between ratios at maximum salivary cortisol concentration and either age ($r = -0.140$, $p = 0.264$), body surface area ($r = -0.044$, $p = 0.727$), or body mass index ($r = 0.010$, $p = 0.940$), and minimum salivary cortisol concentration and either age ($r = 0.132$, $p = 0.290$), body surface area ($r = 0.069$, $p = 0.587$), or body mass index ($r = -0.067$, $p = 0.595$).

The median value at maximum salivary cortisol concentration in boys was 4.0 (range 1.5 to 29.6). The median value at minimum salivary cortisol concentration in boys was 3.2 (range 0 to 40).

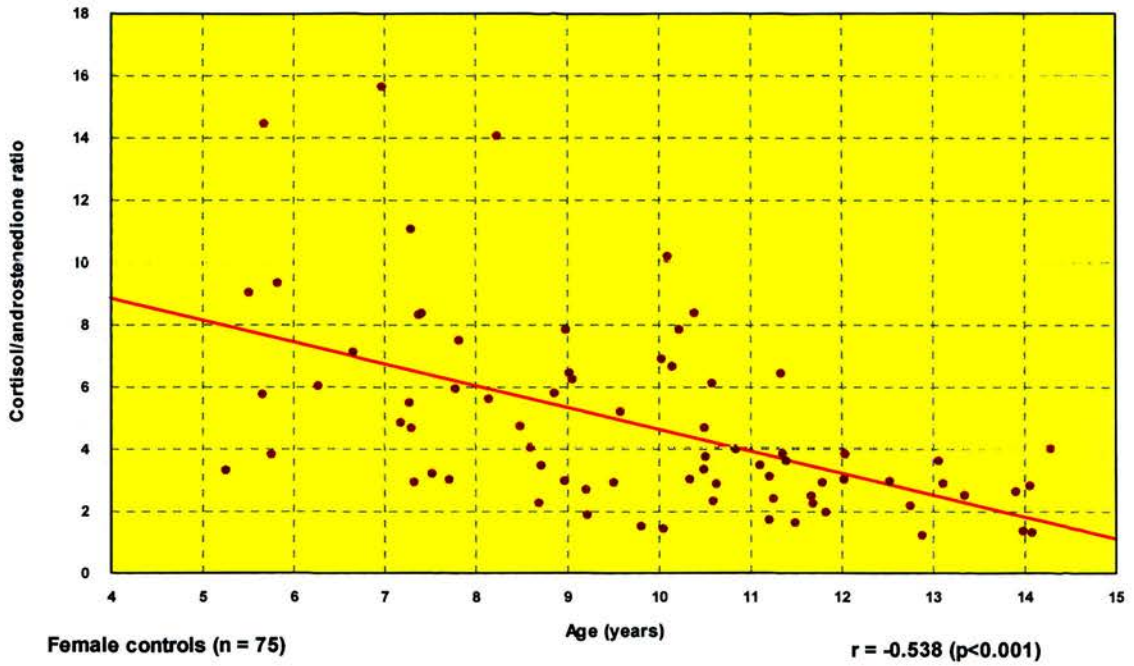

Fig. 6.19. Cortisol/androstenedione ratio against age against maximum salivary cortisol concentration in female controls.

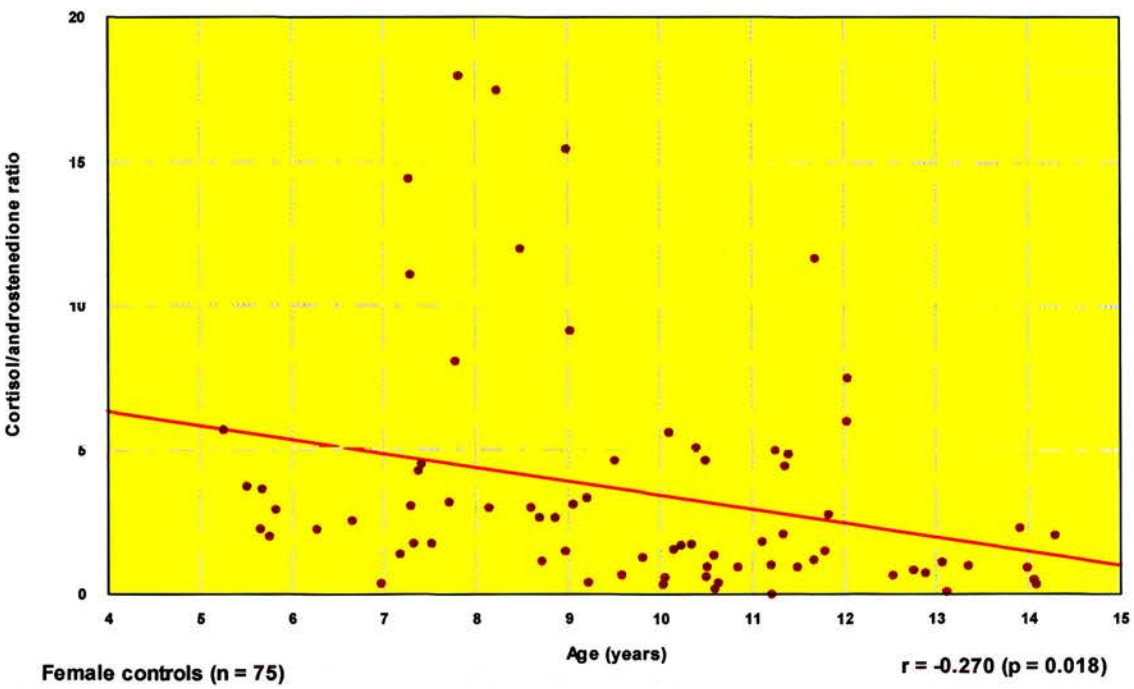

Fig. 6.20. Cortisol/androstenedione ratio against age for minimum salivary cortisol concentration in female controls.

6.3.2.2 Salivary 17-hydroxyprogesterone/Androstenedione Ratios

No correlation was found in boys between salivary 17-OHP/androstenedione ratios and age, body surface area and body mass index.

In girls, there was significant negative correlation between salivary 17-OHP/androstenedione ratios at maximum salivary cortisol concentration and age ($r = -0.397$, $p<0.001$, figure 6.21; c.f. boys: $r = -0.077$, $p = 0.525$, figure 6.22), and body surface area ($r = -0.288$, $p =0.012$; c.f. boys: $r = 0.068$, $p = 0.578$), but not body mass index ($r = -0.187$, $p = 0.106$; c.f. boys: $r = 0.139$, $p = 0.250$).

The median value at maximum salivary cortisol concentration in girls was 0.06 (range 0.01 to 0.3). The median value in boys was 0.05 (range 0 to 0.52).

In girls, there was significant negative correlation between salivary 17-OHP/androstenedione ratios at minimum salivary cortisol concentration and age ($r = -0.459$, $p<0.001$, figure 6.23; c.f. boys: $r = 0.043$, $p = 0.725$, figure 6.24), body surface area ($r = -0.453$, $p<0.001$;c.f. boys: $r = 0.101$, $p = 0.406$), and body mass index ($r = -0.319$, $p = 0.005$; c.f. boys: $r = 0.056$, $p = 0.647$). No such correlation was found in boys.

Using stepwise regression analysis, the main predictor in girls was age for ratios at maximum and minimum salivary cortisol concentration.

The median value at minimum salivary cortisol concentration in girls was 0.19 (range 0.02 to 2.86). The median value in boys was 0.22 (range 0.04 to 0.6).

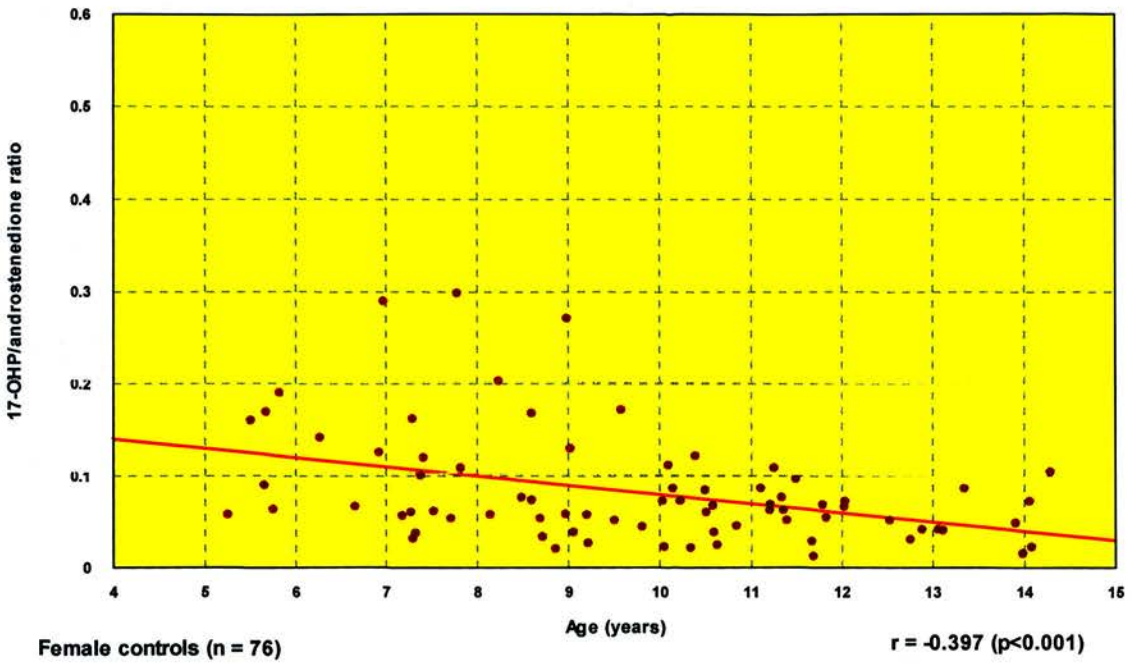

Fig. 6.21. 17-hydroxyprogesterone/androstenedione ratio against age for maximum salivary cortisol concentration in female controls.

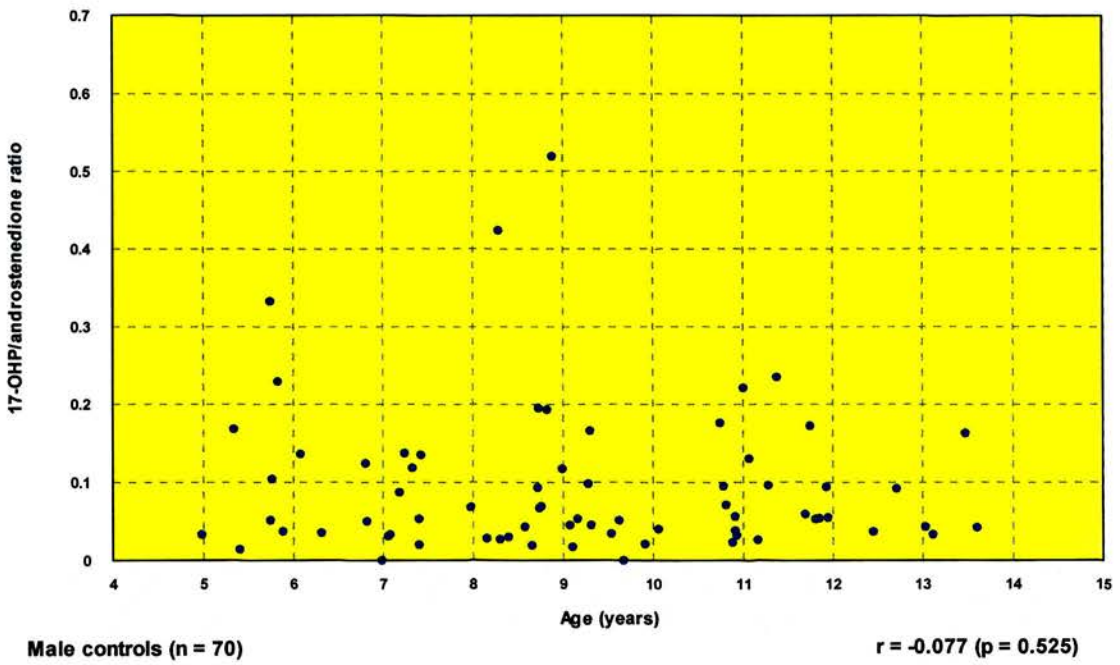

Fig. 6.22. 17-hydroxyprogesterone/androstenedione ratio against age for maximum salivary cortisol concentration in male controls.

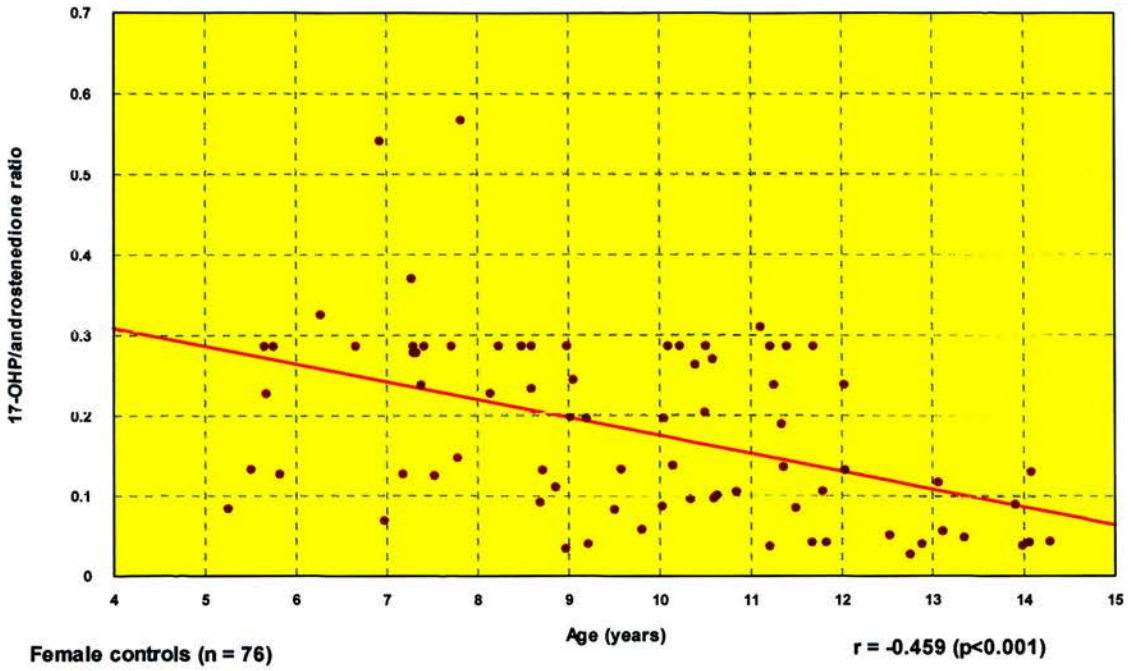

Fig. 6.23. 17-hydroxyprogesterone/androstenedione ratio against age for minimum salivary cortisol concentration in female controls.

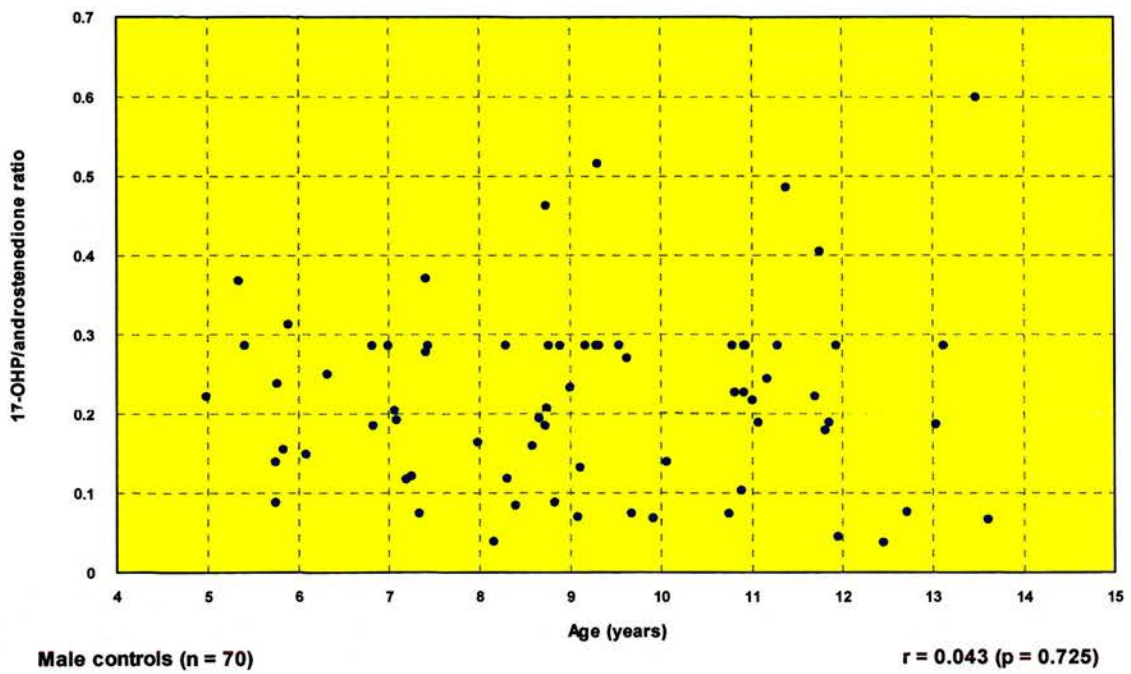

Fig. 6.24. 17-hydroxyprogesterone/androstenedione ratio against age for minimum salivary cortisol concentration in male controls.

6.3.3 Summary of Steroid Profile Results in Controls

◊ All three steroids exhibited a diurnal rhythm, but with a wide variation in salivary steroid concentrations. The minimum concentrations were often undetectable.

◊ No correlation was found between salivary cortisol concentrations and age, body surface area or body mass index, in the age range studied, nor was there a demonstrable sex difference.

◊ A measurable morning salivary cortisol concentration was found in all control profiles.

◊ Both salivary 17-OHP and androstenedione concentrations were higher in girls than in boys.

◊ Salivary 17-OHP and androstenedione concentrations rose with age in girls; statistical correlation was not demonstrated in boys.

◊ It was not possible to determine whether the rise in 17-OHP and androstenedione was gradual or whether it occurred in relation to the onset of adrenarche and puberty.

6.3.4 Summary of Results of Salivary Steroid Ratios

◊ There was no correlation demonstrated for the ratios of salivary cortisol to 17-OHP at both maximum and minimum salivary cortisol concentrations and age, sex, weight or body mass index.

◊ The ratios of cortisol to androstenedione demonstrated a significant negative correlation at maximum and minimum salivary cortisol concentrations in girls with age as the main predictor. This was not the case in boys where no correlation was found.

This suggests that there is a greater rise in androstenedione concentration than cortisol concentration in girls with advancing age.

◊ The ratios of 17-OHP to androstenedione in saliva failed to demonstrate any relationship in boys. However, in girls, there was a significant negative correlation at both maximum and minimum salivary cortisol concentrations for age.

This suggests that there is an increase in androstenedione concentration relative to 17-OHP with advancing age in girls.

6.3.5 Salivary steroids in the study patient group (basal and stressed)

The maximum salivary steroid hormone concentration was compared, as was the minimum salivary steroid concentration, the area under the curve for each steroid hormone profile, and the maximum/minimum ratio of the steroid hormones measured.

6.3.5.1 Salivary Cortisol Profiles (see Figure 6.29 and Tables 6.6 and 6.7)

All except two patients showed a diurnal variation of cortisol in saliva (G05 and B16, Figures 6.25, 6.26 respectively). These patients had flat profiles with concentrations of salivary cortisol below the limit of sensitivity. A further two patients had salivary cortisol concentrations at the lower end of the normal range (B18 and G10, Figs. 6.27 and 6.28 respectively). The remainder of the study patient profiles all had salivary cortisol levels within the reference range of the control children (Fig. 6.29). The median maximum basal salivary cortisol concentration in the study patients (excluding the two abnormal profiles) was 15.7 nmol/L (range 9.2 to 20.5 nmol/L), similar to the control value (median 14.5 nmol/L, range 10.3 to 18.2). The median minimum basal salivary cortisol concentration (excluding the abnormal profiles) was 2.5nmol/L (range 1.7 to 3nmol/L), not different form the control median value of 1.4 (range <0.9 to 16.3 nmol/L).

Table 6.6 shows the raw data of salivary steroid results for the female study patient group and Table 6.7 shows the male study group data.

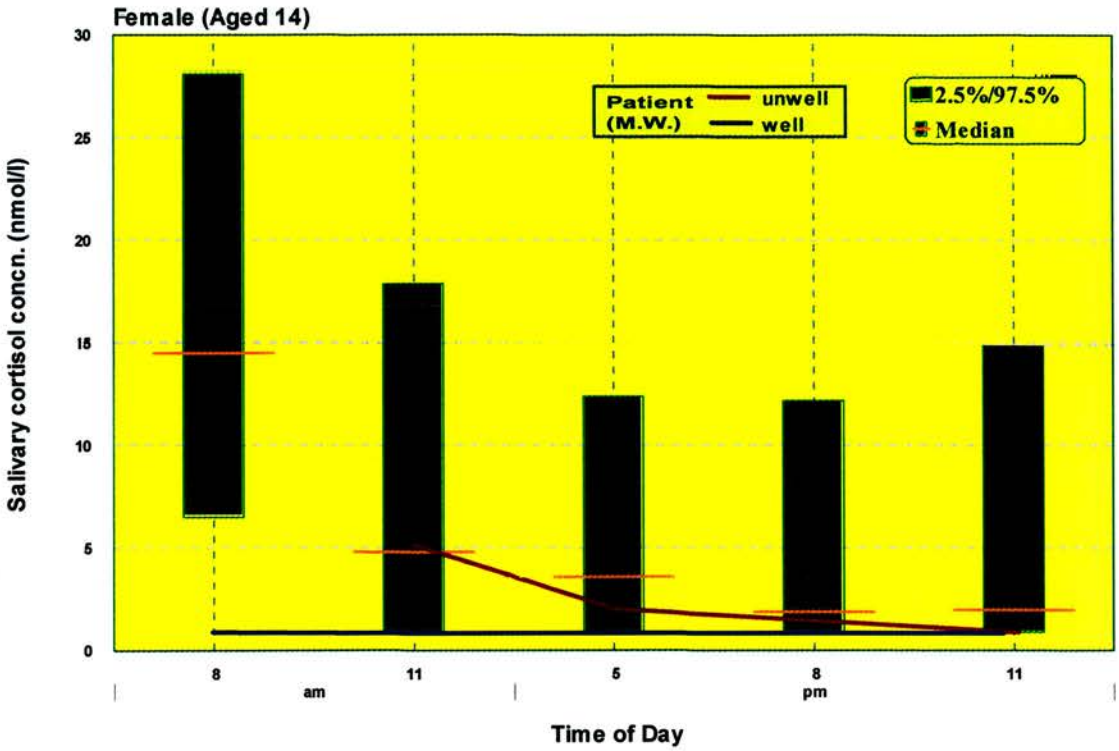

Figure 6.25. Salivary cortisol profiles of a girl (M.W.) with a germ cell tumour of brain.

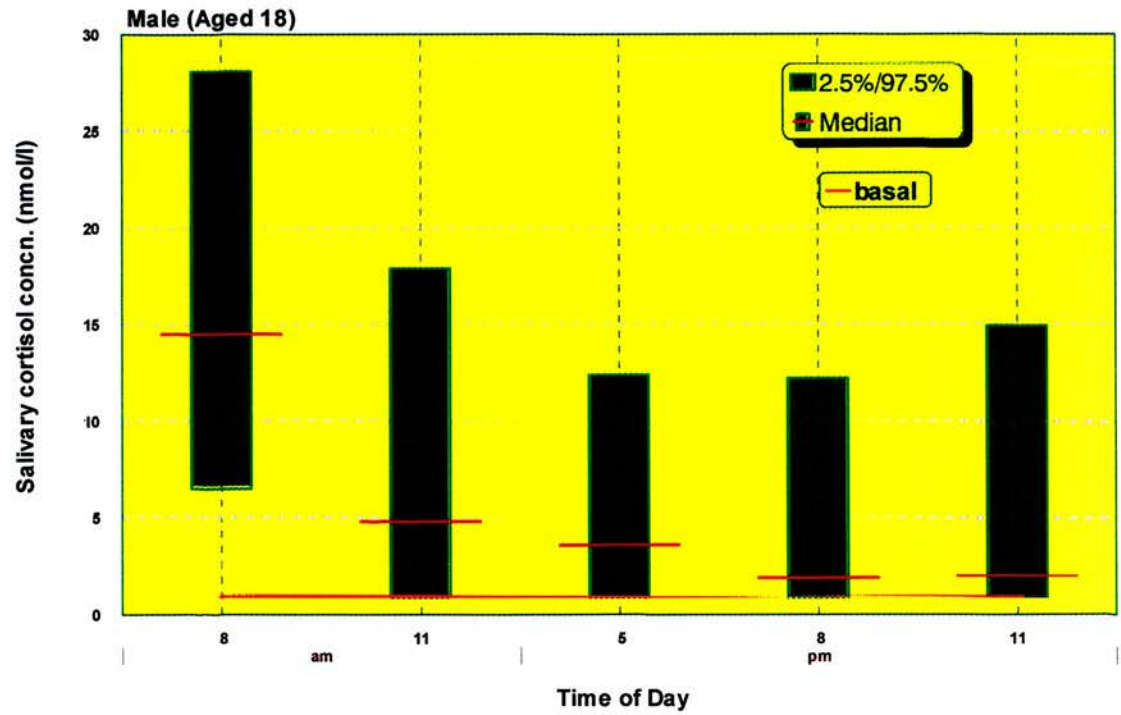

Figure 6.26. Salivary cortisol profile of a boy (C.McQ) with optic glioma.

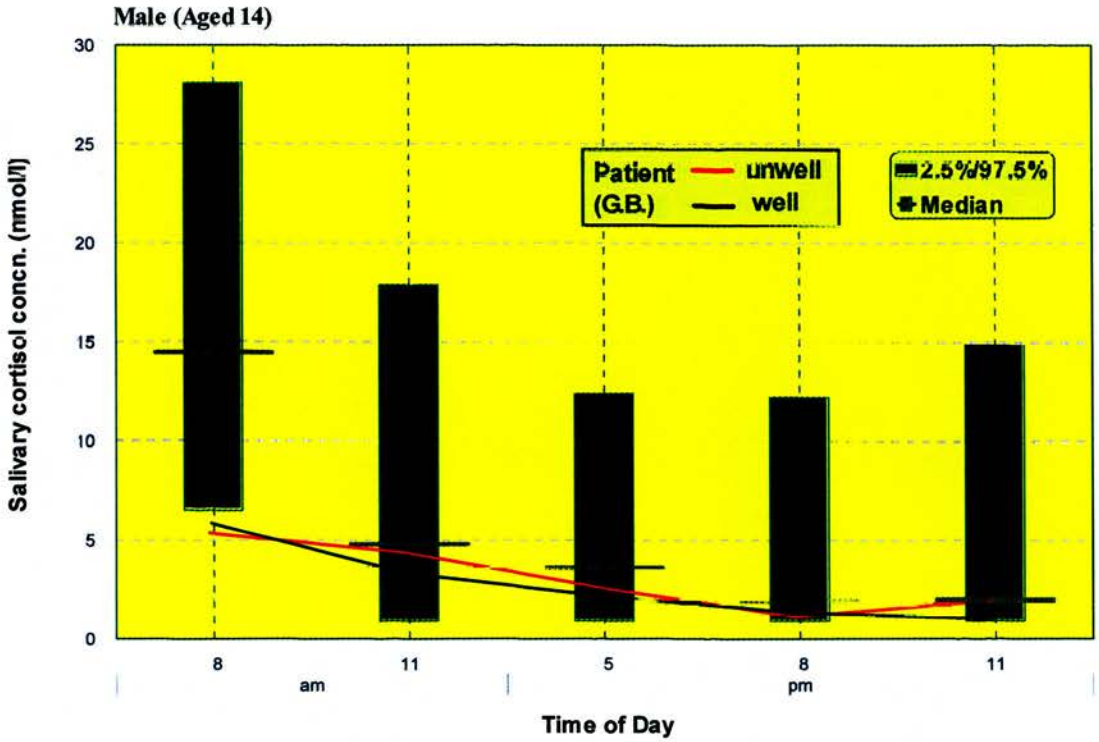

Figure 6.27. Salivary cortisol profiles in a boy (G.B) with a medulloblastoma.

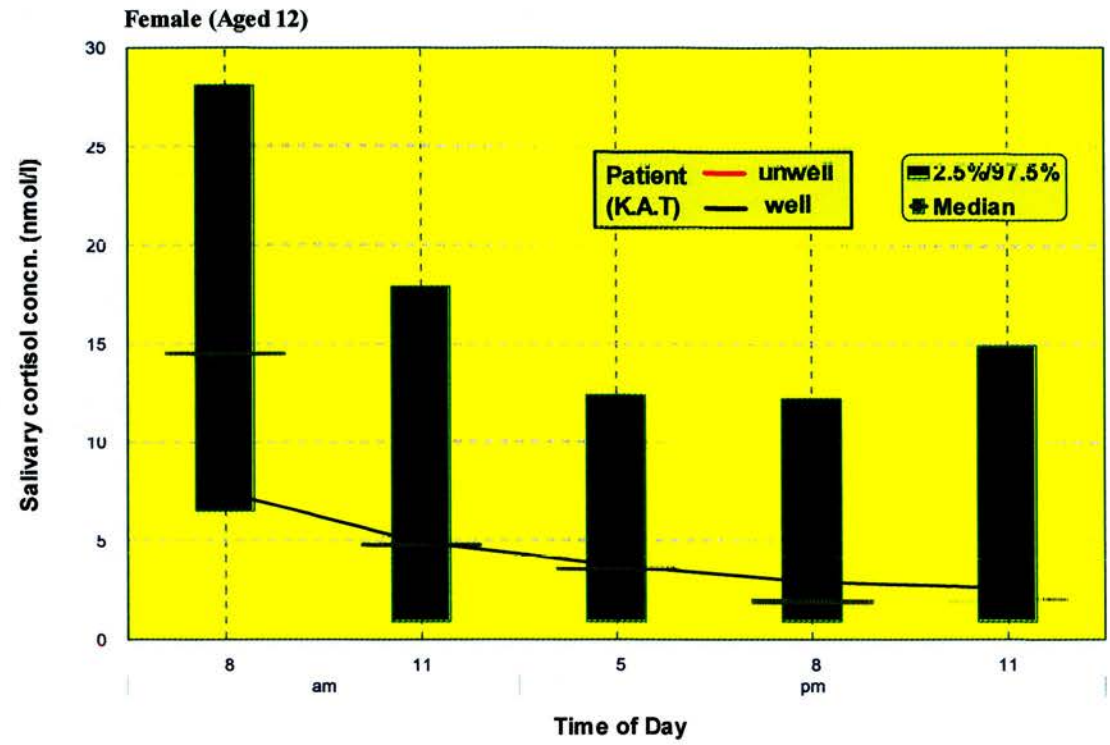

Figure 6.28. Salivary cortisol profiles in a girl (K.A.T) with a medulloblastoma.

		Cortisol					17-OHP					Androstenedione				
No.	Diagnosis	8a.m.	11a.m.	5p.m.	8p.m.	11p.m.	8a.m.	11a.m.	5p.m.	8p.m.	11p.m.	8a.m.	11a.m.	5p.m.	8p.m.	11p.m.
G01	ALL relapse	68	58	63	72	52	0.41	0.29	0.31	0.14	0.1	14.3	6.59	3.61	ND	3.85
G02	TBI - ALL relapse	9.9	8.9	2.8	1.9	2.8	0.19	0.11	0	0.06	0.13	1.53	1.4	0.57	0.15	0.86
G03	TBI - ALL relapse	8.8	4.8	4.2	2.3	1.7	0.21	0.11	0	0	0	6.25	1.98	2.79	1.82	1.9
G04	Ependymoma	13.8	10	10.6	8.3	7.9	0.4	0.27	0.43	0.37	0.63	9.78	6.44	6.77	4.37	6.35
G05	Germinoma	1.3	1	1	1	ND	0.24	0.14	0.17	0.05	ND	0.46	0.23	0.11	0.48	ND
G06	Suprasellar asatrocytoma	7.4	14.2	6.5	8.9	1.9	0.12	0.31	0.2	0.15	0.07	3.76	6.17	4.05	2.71	1.43
G07	Low grade astrocytoma	18.4	10.8	10.1	1.4	0.8	0.33	0.18	0.18	0.17	0.19	5.71	3.68	4.76	3.71	5.23
G08	Optic glioma	23.1	11.8	2.4	33	ND	0.34	0.11	0	0.46	ND	7.62	ND	ND	ND	ND
G09	Optic glioma	8.8	5.2	3.1	2.6	20.2	0.18	0.12	0.14	0.11	0.3	2.97	1.27	1	0.49	5.76
G10	Optic glioma	7.7	5.7	3.8	3	3.6	0.04	0.14	0	0.05	0.18	0.32	ND	ND	ND	1.24
G11	Medulloblastoma	21.3	18.4	7.1	3.5	3.1	0.29	0.25	0.2	0.14	0	10.8	4.63	3.2	1.57	2.11
G12	Medulloblastoma	34	8.5	12.5	2.8	2.9	0.29	0.13	0.22	0.22	0	4.39	3.5	2.44	2.3	2.12
G13	Medulloblastoma	8.1	5.3	12.4	4.4	5.4	0.07	0.12	0.19	0.12	0.09	4.49	2.48	4.33	2.5	2.64
G14	Medulloblastoma	18.3	3.9	3.3	12.7	1.2	0.47	0.09	0.24	ND	0.04	3.92	0.65	0.42	ND	ND
G15	Medulloblastoma	18.4	18.4	38.2	16.3	16.8	0.52	0.91	ND	0.13	ND	2.7	1.3	ND	1.19	ND

(ND = No Data)

Table 6.6. Salivary Steroid Results in Female study patients in adrenal late effects study

		Cortisol					17-OHP					Androstenedione				
No.	Diagnosis	8a.m.	11p.m.	5p.m.	8p.m.	11p.m.	8a.m.	11a.m.	5p.m.	8p.m.	11p.m.	8a.m.	11a.m.	5p.m.	8p.m.	11p.m.
B01	ALL	6.0	9.8	3.6	4.0	1.9	0.4	0.2	0.0	0.0	0.0	3.8	4.6	1.1	1.0	0.5
B02	ALL relapse	20.0	6.0	2.9	3.9	3.6	0.6	0.2	0.1	0.2	0.1	6.2	3.9	2.0	2.5	5.4
B03	ALL relapse	14.3	6.7	ND	5.6	2.9	0.3	0.2	ND	0.2	0.1	3.5	2.8	ND	2.3	1.0
B04	TBI - ALL relapse	18.2	4.5	4.7	2.1	ND	0.2	0.0	0.1	0.2	ND	6.0	3.0	2.0	1.5	ND
B05	TBI - ALL relapse	14.8	4.5	6.8	3.8	2.7	0.3	0.2	0.1	0.1	0.1	6.8	2.6	2.9	1.7	0.4
B06	TBI - ALL relapse	20.7	5.2	3.9	1.6	2.5	0.3	0.3	0.2	0.2	0.1	3.4	1.1	1.5	0.4	0.6
B07	TBI- ALL relapse	9.2	4.9	6.2	3.6	2.6	0.2	0.2	0.2	0.0	0.1	6.5	2.7	2.9	2.5	2.3
B08	TBI-AML	22.1	18.3	10.9	6.2	4.2	0.8	0.7	0.5	0.4	0.5	11.7	8.3	6.1	5.3	8.1
B09	TBI-AML	13.4	7.8	4.7	7.1	4.1	0.0	0.0	0.0	0.1	0.0	1.3	1.6	ND	ND	ND
B10	TBI-AMML	15.6	7.5	6.1	2.7	1.1	0.6	0.3	0.3	0.2	0.2	5.8	4.6	4.3	3.6	3.4
B11	TBI - Neuroblastoma	15.4	4.9	2.8	3.2	2.8	0.2	0.0	0.1	0.0	0.1	4.1	2.1	1.7	0.6	1.1
B12	Lymphoma (B cell NHL)	22.1	10.0	13.5	3.8	1.1	0.4	0.2	0.2	0.2	0.2	3.7	3.0	2.9	1.6	0.8
B13	Brain stem	15.8	3.3	3.4	2.2	ND	0.2	0.1	0.1	0.0	ND	4.9	ND	3.1	1.8	ND
B14	Glioma	18.0	7.0	4.0	3.0	ND	0.3	0.3	0.1	0.2	ND	ND	1.5	ND	0.7	ND
B15	Optic glioma	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
B16	Optic glioma	0.9	0.9	0.9	0.9	0.9	0.1	0.1	0.1	0.1	0.1	1.0	0.6	0.5	0.4	1.6
B17	Germ cell	17.1	9.3	5.9	6.2	3.3	0.1	0.2	0.4	0.3	0.2	4.6	2.3	0.8	1.0	1.2
B18	Medulloblastoma	9.3	5.4	3.6	2.3	1.8	0.3	0.2	0.2	0.2	0.0	ND	ND	ND	ND	ND
B19	Medulloblastoma	22.7	10.2	10.5	0.5	ND	0.3	0.3	0.3	0.3	ND	2.2	1.8	1.6	0.3	ND
B20	Medulloblastoma	22.5	5.2	5.1	1.8	ND	0.3	0.1	0.1	0.3	ND	4.3	2.1	0.4	0.3	ND
B21	Rhabdomyosarcoma of jaw	16.2	10.7	7.9	6.8	7.1	0.2	0.1	0.1	0.1	0.1	2.5	1.4	1.3	ND	0.7

(ND = No Data)

Table 6.7. Salivary Steroid Results in Male study patients in adrenal late effects study

In some patients who had provided basal and stressed salivary profiles, there was a rise in salivary cortisol concentration under stress compared with their basal profiles. This was particularly notable when the maximum morning salivary cortisol concentrations were compared. However, the median maximum stressed salivary cortisol concentration (18.6 nmol/L) was only slightly higher than the median basal value (interquartile range 10.8 to 24.9 nmol/L; Figure 6.30). The unwell:well maximum salivary cortisol ratio varied from 0.6 to 3.0 (median value = 1.2). The median minimum stressed salivary cortisol concentration was 3.4 nmol/L (interquartile range 2.0 - 6.9 nmol/L). This was significantly higher than the median minimum basal values in these patients and higher than the controls (1.4 nmol/L).

Comparing AUC for profiles taken under basal conditions and under stress, the differences between the basal values and stressed values were variable. The unwell:well ratios of area under the curve for salivary cortisol profiles varied from 0.7 to 2.4 (median = 1.2), with only 6 out of 11 paired profiles having ratios of greater than unity.

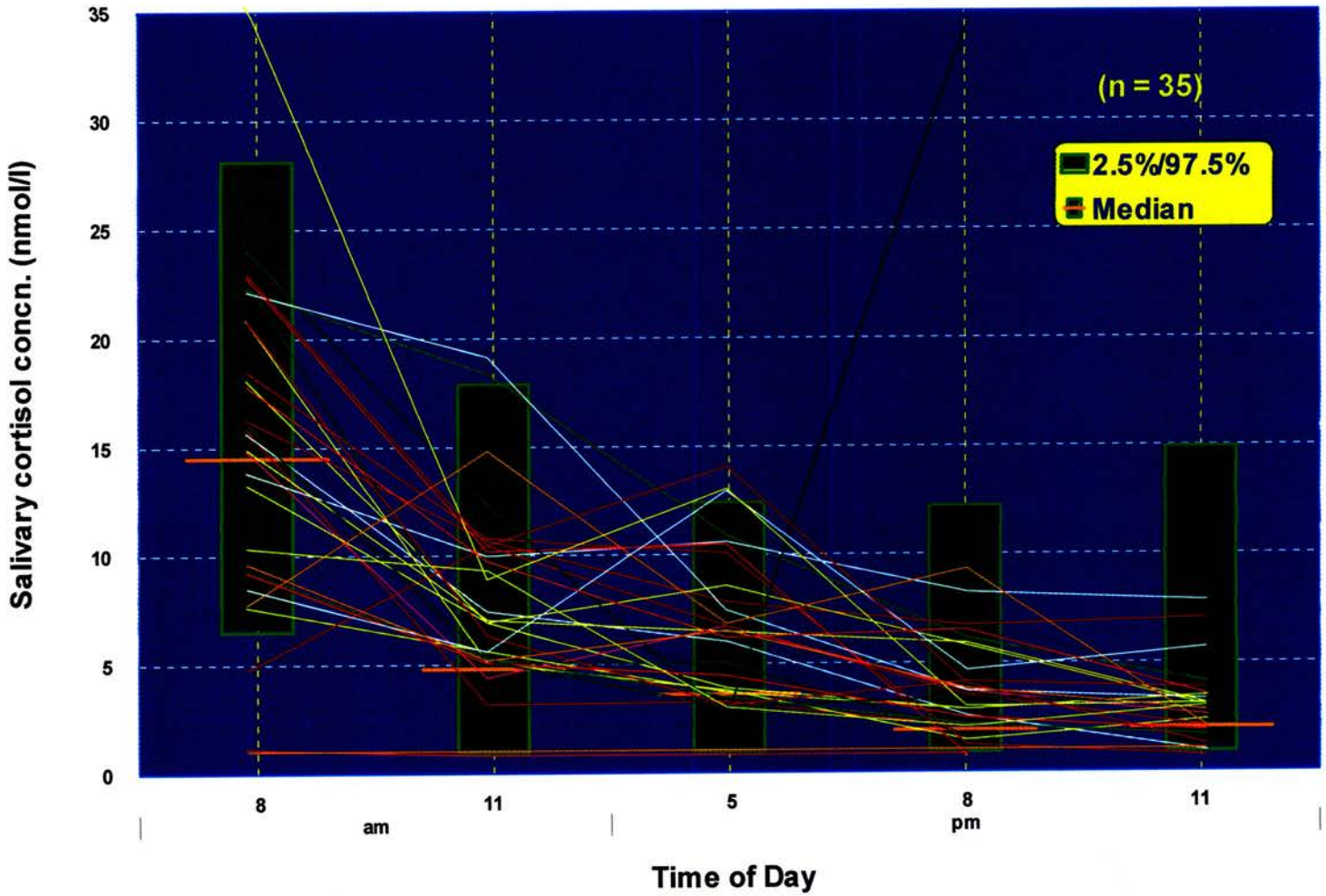

Figure 6.29. Salivary cortisol profiles of all study patients who provided salivary profiles.

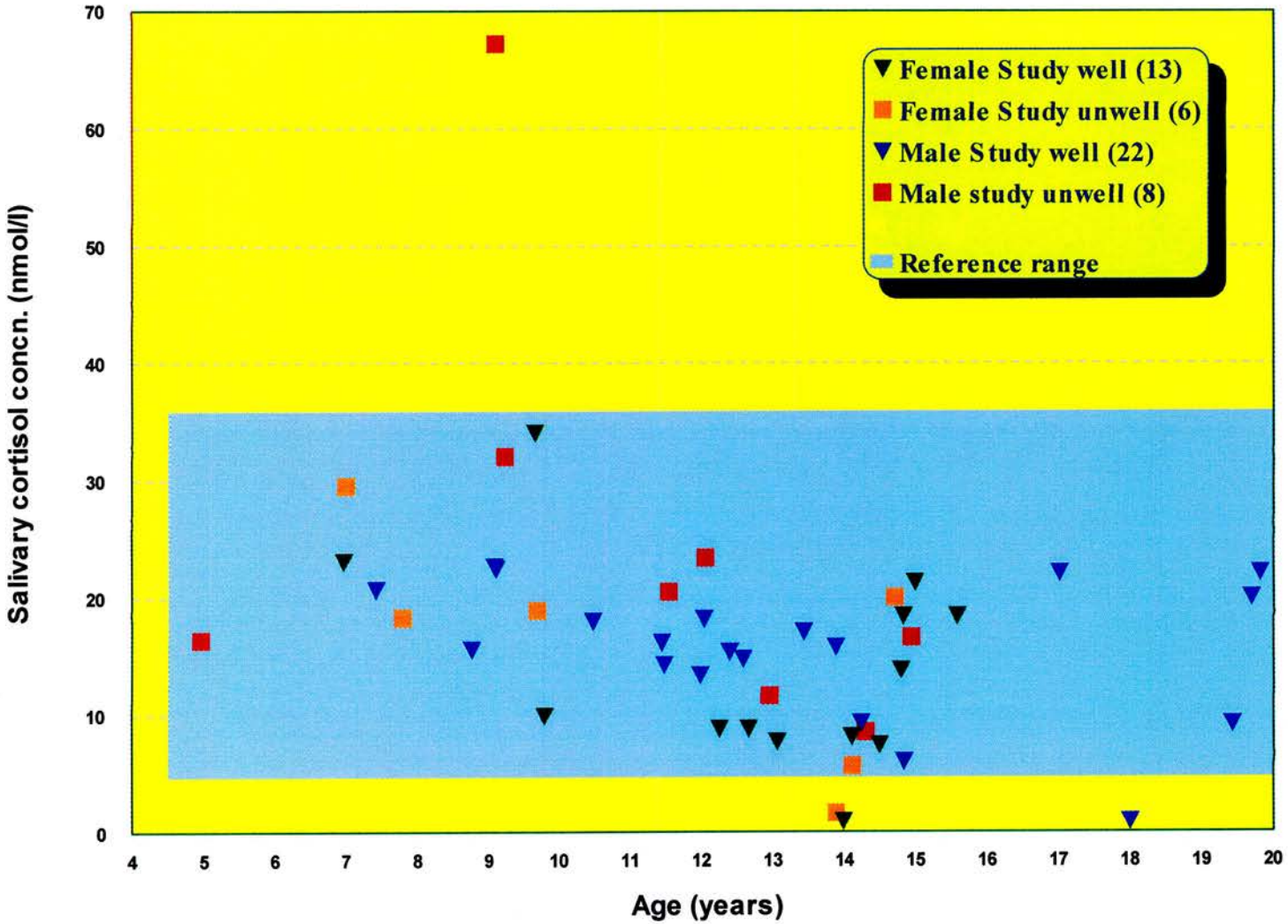

Figure 6.30. Maximum salivary cortisol concentrations: study patient data.

6.3.5.2 Salivary 17-Hydroxyprogesterone Profiles

There was also a diurnal variation in salivary 17-OHP concentration in the 24 hour profiles in the study patients. The median maximum and minimum salivary 17-OHP concentration was similar in girls and boys (Tables 6.6 and 6.7 respectively). Comparison of basal and stressed concentrations of maximum and minimum salivary 17-OHP concentrations showed a rise in 17-OHP levels in response to stress in some but not all of the patients.

In the patients with abnormal salivary cortisol profiles, the 17-OHP profiles did not provide any additional information. The profiles in these patients were either normal or demonstrated low concentrations (particularly in one patient B16).

6.3.5.3 Salivary Androstenedione Concentration

There was also a diurnal variation in salivary androstenedione concentration in the 24-hour profiles in the study patients. The median maximum and minimum salivary androstenedione concentration was higher in girls than boys.

Comparison of basal and stressed concentrations of maximum and minimum salivary androstenedione concentrations showed lower in androstenedione concentrations in response to stress in the female patients, but not in male patients.

Tables 6.6 and 6.7 show the salivary androstenedione concentrations in the female and male study patient group.

As with the 17-OHP profiles, the androstenedione profiles did not provide any additional information regarding the likelihood of being hypoadrenal in the patients with low salivary cortisol profiles.

6.3.5.4 Salivary Cortisol/17-Hydroxyprogesterone Ratios

The ratio of salivary cortisol to salivary 17-OHP was calculated at the maximum and minimum salivary cortisol concentrations. The median value (and interquartile range) for ratios at maximum basal salivary cortisol concentration for the study group, control girls and control boys was 61.30 (41.90 to 79.10), 55.6 (42.6 to 77.0), and 57.5 (41.7 to 91.4) respectively. The median ratio at maximum salivary cortisol concentration for stressed profiles in the study group was 41.0 (interquartile range 22.9 to 86.7). Although this was lower than with the basal levels, there was more of a spread of results; the interquartile range was 22.90 to 86.70.

The median ratios (and interquartile range) at minimum basal salivary cortisol concentrations for the study group, control girls and control boys was 19.0 (9.0 to 31.10), 12.0; interquartile range 9.0 to 21.0). For stressed levels in study patients the median value was 43.65 (interquartile range 14.10 to 69.30).

6.3.5.5 Salivary Cortisol/Androstenedione Ratios

The ratio of salivary cortisol to salivary androstenedione was calculated at the maximum and minimum salivary cortisol concentrations. The median value (and interquartile range) for ratios at maximum basal salivary cortisol concentration for the study group, control girls and control boys was 31.0 (18 to 61), 21.6 (9.6 to 45), and 31.9 (14.6 to 56.6) respectively. The median ratio at maximum salivary cortisol concentration for stressed profiles was 44 (interquartile range 34 to 95). The median value (and interquartile range) for ratios at minimum salivary cortisol concentrations for basal levels in the study group, control girls and boys was 15 (10 to 28), 21.6 (9.6 to 45) and 31.9 (14.6 to 56.6) respectively). For stressed levels the median ratio was 38 (interquartile range 1.0 to 63).

6.3.5.6 Salivary 17-Hydroxyprogesterone/Androstenedione Ratios

The ratio of salivary 17-OHP to salivary androstenedione was calculated at the maximum and minimum salivary cortisol concentrations. The median ratio at maximum salivary cortisol concentration was 0.59 (interquartile range 0.28 to 0.97). (C.f. controls: median; girls = 0.64; boys = 0.55; interquartile range 0.45 to 1.0, and 0.34 to 1.3 respectively). The median ratio at maximum salivary cortisol concentration for stressed profiles was 0.91 (interquartile range 0.74 to 1.61). The median ratio at minimum salivary cortisol concentrations for the basal values was 0.97 (interquartile range 0.52 to 1.86) (C.f. controls: median; girls = 1.9, boys = 2.2; interquartile range 0.9 to 2.9, and 1.2 to 2.9 respectively) and for the stressed levels was 1.02 (interquartile range 0.76 to 1.26).

6.3.5.7 Salivary Steroid Ratios - correlations in study patients

6.3.5.7.1 Ratios at Maximum Salivary Cortisol Concentration

There was a significant correlation between salivary cortisol/17-OHP ratios and salivary cortisol/androstenedione ratios for both basal and stressed values. The correlation coefficient was 0.450 ($p = 0.014$) for basal values and 0.804 ($p<0.001$) for stressed values. There was no correlation found between any of the other ratios calculated.

6.3.5.7.2 Ratios at Minimum Salivary Cortisol Concentration

There was a significant negative correlation between basal salivary cortisol/17-OHP ratios and basal salivary 17-OHP/androstenedione ratios, with a correlation of -0.363. However, this was at the limit of significance, with a p value of 0.049.

There was a significant correlation between basal salivary cortisol/androstenedione ratios and basal 17-OHP/androstenedione ratios, with a correlation coefficient of 0.519 ($p =0.002$).

There was reasonable correlation between stressed salivary cortisol/17-OHP ratios and stressed salivary cortisol/androstenedione ratios ($r =0.704$, $p =0.023$).

6.4 Capillary blood spot profiles in study patients - Basal and Stressed

Thirty five study patients provided blood spot profiles, of which twenty four profiles (69%) were suitable for use. Eleven profiles were unsatisfactory due to insufficient amounts of blood on the filter paper discs and were excluded. The good quality profiles showed a diurnal variation and also an increase in steroid levels in response to stress.

The blood spot profiles could not be directly compared with control data as these were not available. Comparison of blood spot profiles with salivary profiles showed a correlation coefficient of 0.763 ($p<0.0001$) when the results from all patients were pooled together (Figure 6.31). However, there was considerable variation in the correlation of blood spot and salivary profiles for each patient, correlation coefficient varying between 0.656 and 0.997 (e.g. B20 Figure 6.32).

The factor expressing the difference between saliva and capillary blood was calculated and found to be 11.86.

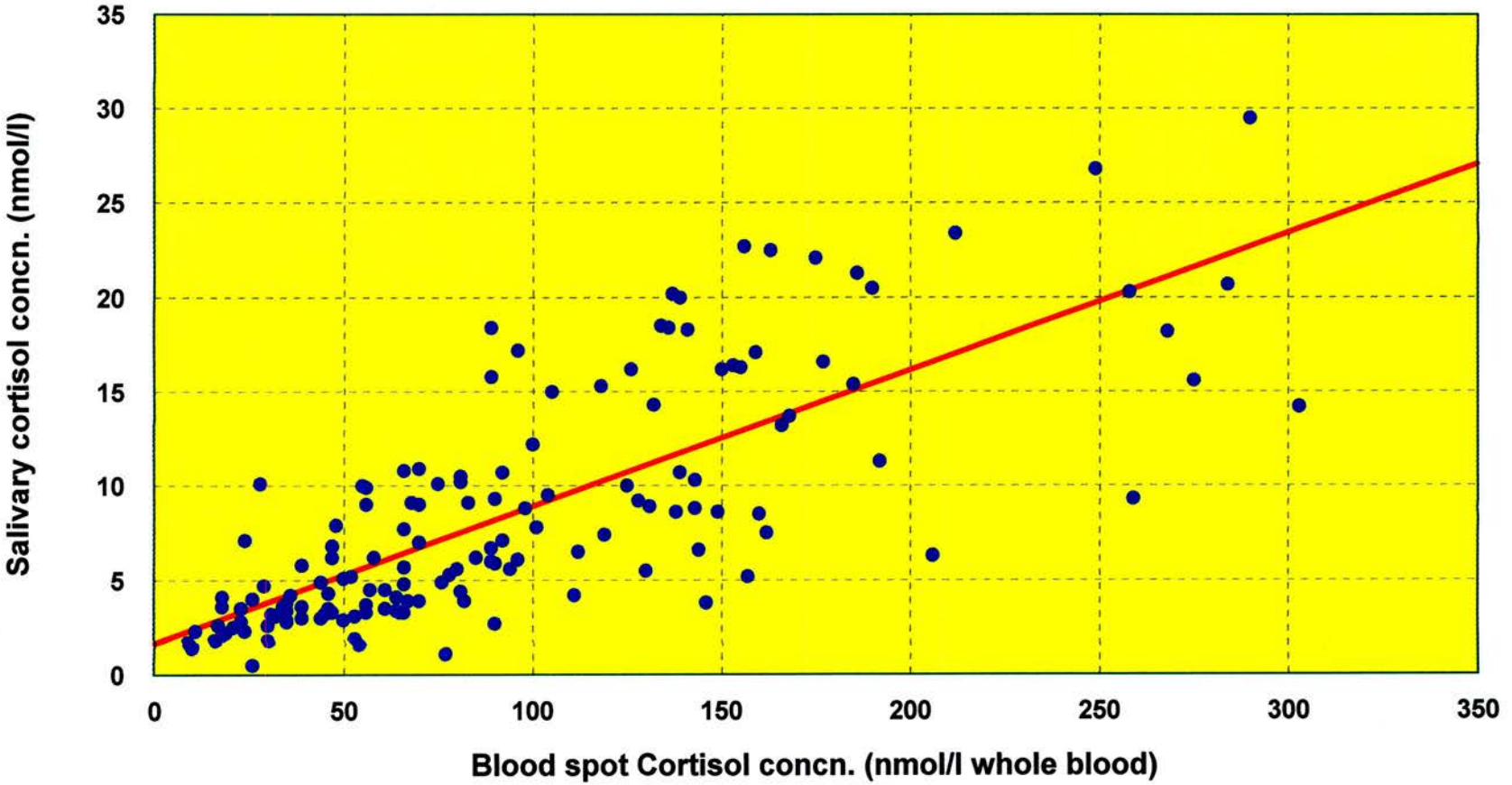

Figure 6.31. Correlation between salivary cortisol concentration and whole blood cortisol measured in capillary blood spot samples in study patients.

6.4.1 Blood Spot Cortisol in Study Patients

From the twenty four satisfactory profiles, the median peak morning cortisol concentration was 155 nmol/L (range 89 - 284 nmol/L). The median minimum cortisol concentration in these profiles was 42.5 nmol/L (range <20 to 200 nmol/L). The limit of sensitivity of the assay was 20 nmol/L of whole blood.

Eight patients provided blood spot profiles taken when they were unwell. Of these, only five were suitable due to poor quality of the samples taken.

The median maximum morning capillary blood spot cortisol concentration in the unwell profiles was 212 nmol/L (range 138 to 364 nmol/L). The median minimum capillary blood spot cortisol concentration was 62 nmol/L (range <20 to 105 nmol/L). Thus, there was only a slight difference in these values between the well and unwell profiles.

The median value for AUC was 2133 for the baseline profiles (range 1199 to 4264), compared with the unwell profiles in which the median value was 3162 (range 1511 to 7157). This shows that cortisol release is generally increased in stress, with increases in peak morning values as well as daytime levels.

The results of the capillary blood spot profiles in the study patients is shown in Table 6.8.

6.4.2 Blood Spot 17-Hydroxyprogesterone in Study Patients

The limit of sensitivity of the 17-OHP assay was 2 nmol/L.

Eighteen baseline profiles were available for 17-OHP measurement in blood spots, five profiles were performed in study patients when they were unwell.

The median maximum 17-OHP concentration in the well study patient profiles was 8.1 nmol/L (range 3.9 to 30.9 nmol/L). The median minimum value was <2 nmol/L

(range <2 to 4.2 nmol/L). In the profiles performed in patients when they were unwell, the median maximum 17-OHP concentration was 5.9 nmol/L (range <2 to 13 nmol/L). The median minimum 17-OHP concentration was <2 nmol/L (range <2 to 2.3 nmol/L). The AUC in the well and unwell profiles was 88.9 (range 39.9 to 503) and 99 (range 45 to 108) respectively. These data suggest that under stress, the 17-OHP concentrations actually diminish. (See Table 6.8 for the 17-OHP blood spot results in the study patients).

6.4.3 Blood Spot Androstenedione in Study Patients

Seventeen baseline blood spot profiles were suitable for interpretation. The limit of sensitivity of the androstenedione assay was 0.1 nmol/L.

The mean maximum androstenedione concentration in these baseline profiles was 9.6 nmol/L (range 4.3 to 25.2 nmol/L), compared with 8.6 nmol/L in the stressed profiles (range 2.5 to 11.6 nmol/L). The mean minimum concentration of androstenedione in blood spots was 3.7 nmol/L (range <0.1 to 9.5 nmol/L). In the stressed profiles (five in total), the mean minimum androstenedione concentration was 2.5 nmol/L (range <1 to 4.4 nmol/L).

The AUC for the baseline profiles was 142.2 (range 63.6 to 365). It was not possible to calculate AUC in the stressed profiles as there was insufficient data available.

The raw data for the androstenedione blood spot results in the study patients is shown in Table 6.8.

Female study patients in adrenal late effects study																
		CAPILLARY BLOOD SPOT														
		Cortisol					17-OHP					Androstenedione				
No.	Diagnosis	8a.m.	11a.m.	5p.m.	8p.m.	11p.m.	8a.m.	11a.m.	5p.m.	8p.m.	11p.m.	8a.m.	11a.m.	5p.m.	8p.m.	11p.m.
G01	ALL relapse	1000	640	177	997	234	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G02	TBI - ALL relapse	116	81	35	34	45	4.4	2.3	<2	<2	<2	6.6	0	0	0	4.4
G03	TBI - ALL relapse	144	66	36	11	9	5	<2	<2	<2	<2	ND	4.4	2.8	3	4.9
G04	Ependymoma	214	328	93	124	152	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G05	Germinoma	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G06	Suprasellar asatrocytoma	130	303	112	131	53	7.6	10.1	2.5	ND	<2	ND	ND	3.7	8	ND
G07	Low grade astrocytoma	153	66	28	10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G08	Optic glioma	290	130	149	70	ND	10.8	<2	<2	<2	ND	5.4	ND	1.8	3.1	ND
G09	Optic glioma	143	60	53	17	137	6.1	ND	2.1	<2	8.9	ND	ND	ND	ND	ND
G10	Optic glioma	138	69	57	18	19	5.6	4.3	3.2	2.3	4.2	10.4	8	5.1	3.1	ND
G11	Medulloblastoma	186	136	92	46	32	3.8	<2	4.8	<2	>2	ND	ND	ND	ND	ND
G12	Medulloblastoma	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G13	Medulloblastoma	107	68	97	83	200	6.3	4.3	4.6	2.6	9.8	4.3	3.3	5.4	3.6	7.3
G14	Medulloblastoma	271	403	214	80	71	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
G15	Medulloblastoma	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Male study patients in adrenal late effects study																
		CAPILLARY BLOOD SPOT														
		Cortisol					17-OHP					Androstenedione				
No.	Diagnosis	8a.m.				11p.m.	8a.m.				11p.m.	8a.m.				11p.m.
B01	ALL	176	110	86	76	49	9.3	7.7	<2	<2	<2	8.5	8.6	6.1	4.1	1.8
B02	ALL relapse	139	89	50	67	34	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
B03	ALL relapse	132	89	114	94	35	<2	<2	>2	>2	<2	7.4	6	ND	3.6	1.7
B04	TBI - ALL relapse	268	61	29	18	-	7.3	1.5	0	1.2	ND	5.6	0	0	2	ND
B05	TBI - ALL relapse	182	86	84	49	38	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
B06	TBI - ALL relapse	284	157	82	54	21	17.7	5.4	2.4	<0.5	1.5	6.3	4.4	3.9	1.5	0
B07	TBI- ALL relapse	138	76	85	39	30	13.5	2.4	2.7	2.2	<2	13.7	ND	ND	ND	4
B08	TBI-AML	175	141	70	58	111	30.9	37.2	12	9.2	12.7	25.2	24.4	11.3	5.5	8.5
B09	TBI-AML	125	48	47	35	18	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
B10	TBI-AMML	275	162	96	90	77	23.5	12.6	14	14.8	10.5	18.4	11.6	13.9	9.5	8.7
B11	TBI - Neuroblastoma	185	44	35	31	23	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
B12	Lymphoma (B cell NHL)	155	185	163	76	52	7	4.5	2.2	4.5	2.4	12.7	13	9.6	5.3	4.6
B13	Brain stem	89	56	35	19	ND	6.7	2.1	1.9	<2	ND	4.4	3.3	2.9	2.9	ND
B14	Glioma	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
B15	Optic glioma	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
B16	Optic glioma	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
B17	Germ cell	159	90	90	47	47	8.3	3	<2	<2	<2	6	7.5	6	5	2.5
B18	Medulloblastoma	259	82	20	10	30	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
B19	Medulloblastoma	156	81	81	26	ND	1.1	1.7	0.8	0.5	ND	ND	2.1	0	0	ND
B20	Medulloblastoma	163	52	50	16	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
B21	Rhabdomyosarcoma of jaw	150	92	48	47	24	7	<2	<2	<2	<2	6.4	ND	ND	ND	2.6

(ND = No Data)

Table 6.8. Blood spot steroid profile results in study patients.

6.4.4 Blood Spot Cortisol/17-hydroxyprogesterone Ratios in Study Patients

The cortisol/17-OHP ratio was calculated for the maximum morning cortisol concentration in blood spot samples from the study patients. In 12 samples, the median ratio was 16.9 (range 1.3 to 48.9). The ratio of cortisol/17-OHP at minimum cortisol concentrations in blood spot samples was little different at 16 (range 4.5 to 26.5). Ratios of cortisol/17-OHP throughout the day were very variable, suggesting no particular pattern for cortisol and 17-OHP concentrations.

6.4.5 Blood Spot Cortisol/Androstenedione Ratios in Study Patients

Cortisol/androstenedione ratios were calculated in blood spot samples at maximum and minimum blood spot cortisol concentrations.

The median cortisol/androstenedione ratio at maximum cortisol concentration was 15.1 (n=11; range 0.2 to 47.9). and at minimum cortisol concentration, 12.2 (n=10; range 1.8 to 210). Again, the median values were comparable, but the ratios in the samples taken throughout the day were widely varying, with no particular pattern.

6.4.6 Blood Spot 17-Hydroxyprogesterone/Androstenedione Ratios in Study Patients

Ratios for 17-OHP and androstenedione were also calculated in 11 study patients. At the maximum morning cortisol concentration, the median ratio was 1.5 (range 0.5 to 66). At minimum cortisol concentrations, the median ratio was 1.0 (range 0.4 to 15). The mean ratios of these steroid hormones throughout the day were not as widely varying as with the previous ratios calculated, suggesting a certain degree of stability in the concentrations of these hormones throughout the day.

6.5 Low Dose (500ng/1.73m^2) Synacthen test in Study Patients

Forty one study patients were subjected to a low dose synacthen test as part of the assessment of adrenal function. All tests were performed between 09:00 and 11:00 hours. Patients were fasted from midnight the night before the test. Each patient was given a dose of 500 ng synacthen/1.73m^2 body surface area and samples were taken as described earlier.

The criteria for a normal test was based on the accepted criteria for a normal response to the standard synacthen test (a peak cortisol response to standard dose of synacthen (250μg) of greater than 500 nmol/L, an incremental rise of greater than 200 nmol/L, and a basal cortisol of greater than 225 nmol/L).

Nine patients had a basal cortisol concentration of less than 225 nmol/L (median basal cortisol 315.5 nmol/L, range 27 to 1038 nmol/L). However, only 3 patients had a maximum cortisol response of less than 500 nmol/L (median 730 nmol/L; range 58 to 1640 nmol/L). (See figure 6.33) Eight patients had an incremental rise of less than 200 nmol/L (median 369 nmol/L; range 31 to 818 nmol/L. (Figure 6.34) Fifteen patients had at least one abnormal criteria (summarised in Table 6.9).

There was a significant correlation between basal cortisol concentration and peak cortisol concentration ($r = 0.64$, $p<0.001$). There was also a correlation between peak cortisol concentration and incremental rise of cortisol in response to synacthen ($r = 0.64$, $p<0.001$).

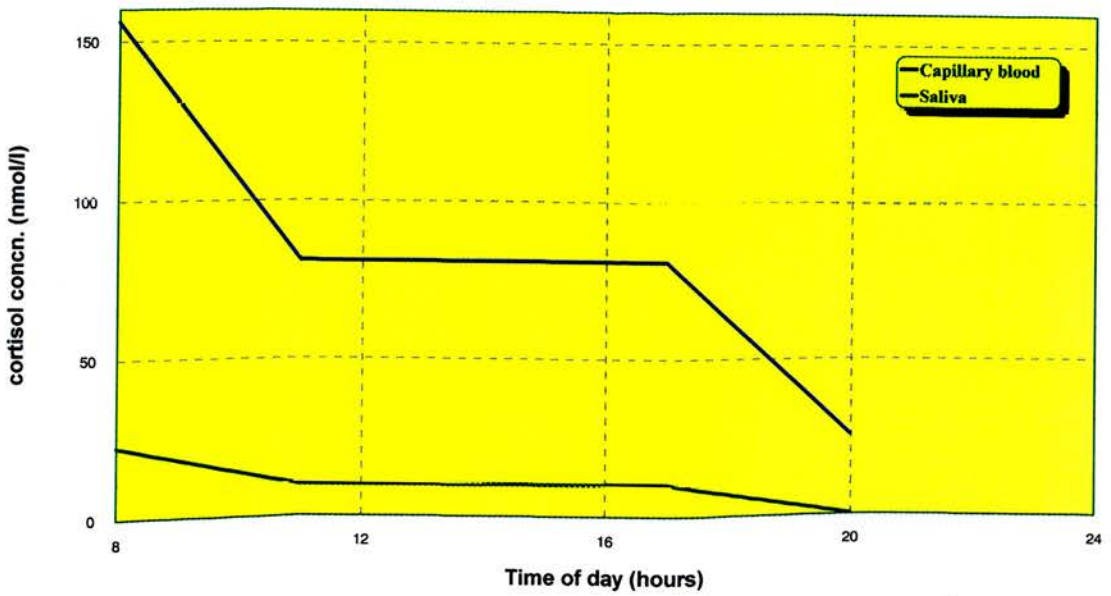

Figure 6.32. Correlation between salivary cortisol and blood spot cortisol in one patient (C.C. Aged 12).

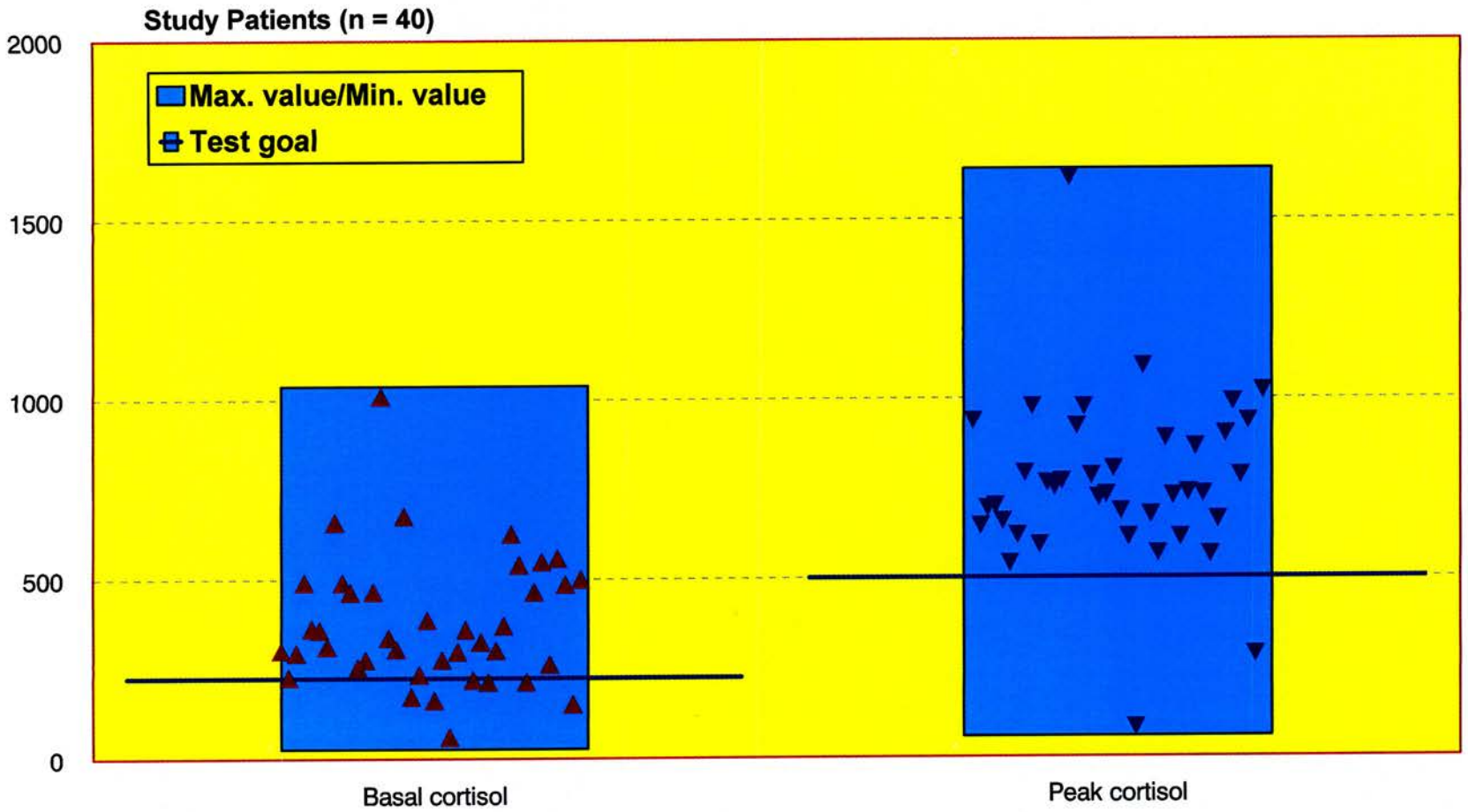

Figure 6.33. Basal and peak cortisol concentrations in study patients undergoing the low dose synacthen test.

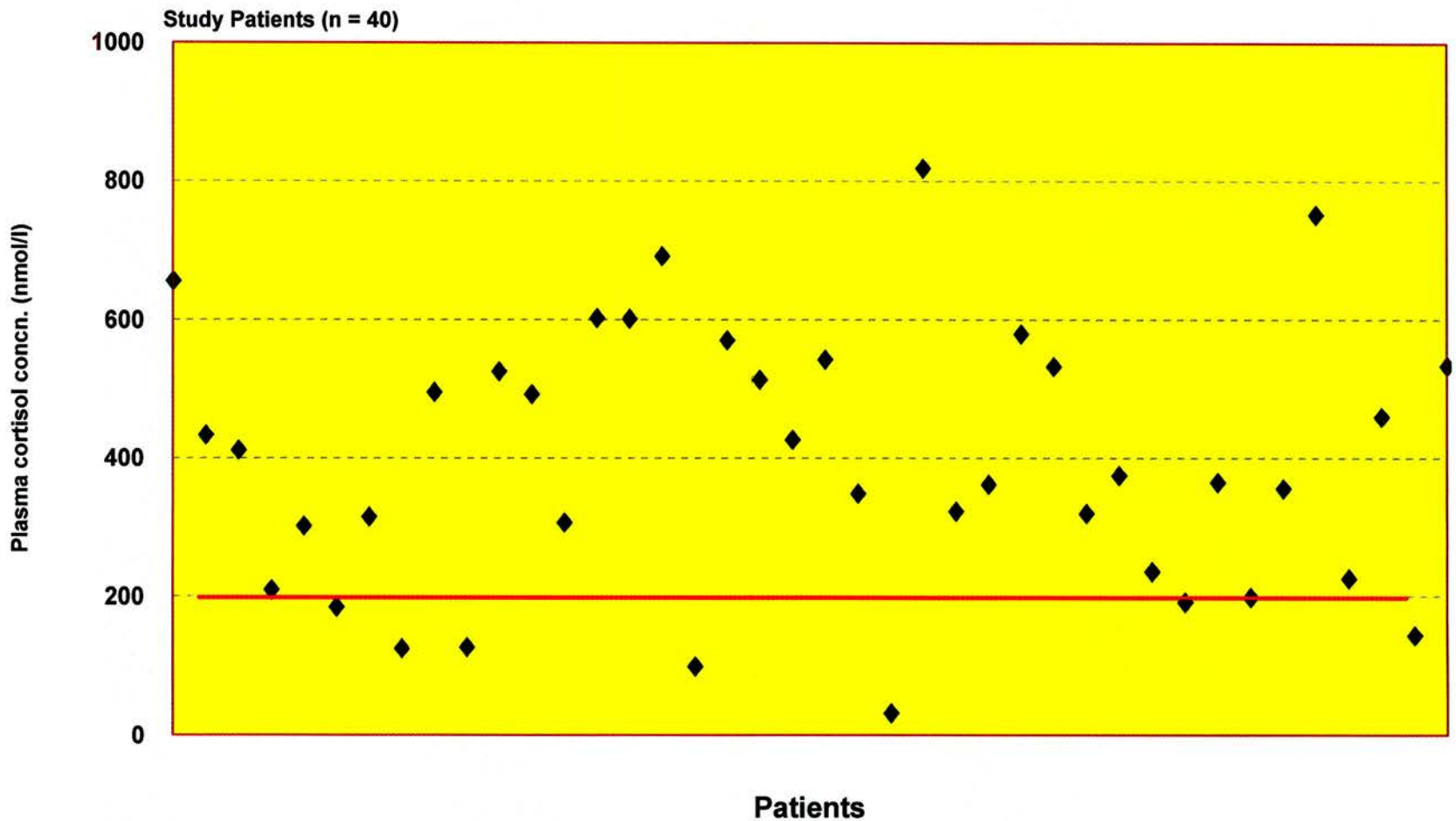

Figure 6.34. Chart showing incremental rise in plasma cortisol in the low dose synacthen test.

Basal cortisol <225nmol/l	Peak cortisol <500nmol/l	Incremental rise <200nmol/l	No. of patients who failed at least one of the criteria
10 (25%)	2 (5%)	8 (20%)	15 (37.5%)

Table 6.9. Analysis of abnormal results of low dose ACTH test in Study patients (n = 41).

6.6 Insulin-hypoglycaemia Results in Study Patients

Of the patients studied, only 6 had an insulin hypoglycaemia test performed during the period of the study. The remainder of the patients who had undergone an ITT had the test performed over a year previously.

In total, 55 patients had an ITT performed, either as part of a routine investigation post transplant or post treatment for brain surgery and radiotherapy, or because of a clinical suspicion of growth hormone deficiency. None of the patients had an ITT performed purely to investigate possible adrenocortical insufficiency.

None of the patients who underwent an insulin hypoglycaemia provocation test were shown to have a subnormal cortisol response to hypoglycaemia.

6.7 Urinary Metyrapone Test Results in Study Patients

A urinary metyrapone test was performed in five patients in whom there was a high index of clinical suspicion of ACTH deficiency.

The test involved the patient collecting urine over 24 hours as an initial baseline collection, following which oral metyrapone was given in a low dose (500 mg/m^2). Metyrapone is an 11β-hydroxylase inhibitor, which reduces cortisol concentration by blocking its synthesis (see Figure). Steroids (11-deoxy) immediately prior to the block are increased as a result of increased ACTH drive. Following administration of metyrapone, two consecutive 24 hour urine collections were performed. By measuring the ratios of cortisol urinary metabolites to 11-deoxycortisol metabolites, and corticosterone to 11-deoxycorticosterone, ACTH deficiency can be assessed. (218).

From the five patients tested, two patients had an abnormal metyrapone challenge. These patients also had symptoms of SAI, which were greatly exacerbated by the single dose of metyrapone. (See Tables 6.11; 6.12).

Normal data	THB	THA		THF	THS
15 males, 4 females age 4-10 years	ud-121	ud-131		185-649	<5
6 males age 11-15 years	ud-34	ud-36		52-181	<5

units = µg/24 hours

Table 6.10. Normal values for urinary steroids (cortisol and its metabolites)

		Day 1						Day 2						Day 3					
Patient	Sex	THB	THA	THB/THA	THF	THS	THF/THS	THB	THA	THB/THA	THF	THS	THF/THS	THB	THA	THB/THA	THF	THS	THF/THS
M.W.	F	ud	100	_	210	ud	_	130	110	1.18	240	370	0.65	ud	ud	_	140	140	1.00
K.D.	F	ud	ud	_	1520	210	7.2381	ud	ud	_	1350	770	1.75	ud	ud	_	1090	740	1.47
C.S.	F	ud	ud	_	780	ud	_	250	700	0.36	1290	1450	0.89	_	_	_	_	_	_
J.N.	M	120	180	0.67	990	180	5.5	130	240	0.54	950	1120	0.85	100	150	0.67	840	320	2.63
C.McQ.	M	ud	ud	_	210	ud	_	ud	ud	_	ud	ud	_	ud	ud	_	ud	ud	_

THB = Tetrahydrocorticosterone
THA = Tetrahydro-11-dehydrocorticosterone
THF = Tetrahydrocortisol
THS = Tetrahydro-11-deoxycortisol
ud = undetectable
units = µg/24 hours

Table 6.11. Results of 3 day urinary metyrapone test on five study patients

6.8 Diagnostic Breakdown of Adrenal Status based on the Results in the Study Patients

6.8.1 Normal

Patients with normal adrenocortical function were classified as those with a salivary cortisol profile within the reference range and with a normal response to the short synacthen test. These patients were also asymptomatic in terms of having no symptoms or clinical features which could have been attributable to adrenocortical insufficiency, and, where applicable, a normal cortisol response to insulin hypoglycaemia, and normal metyrapone test.

Of the patients in the study, 26 patients were found to have normal function according to these criteria.

6.8.2 Borderline

Patients with a borderline result from the above tests were those in which one of the tests performed (either the salivary profiles or the low dose synacthen test) was abnormal. The low dose Synacthen test gave equivocal results in 13 out of 41 patients in whom the test was performed. In all patients with equivocal low dose synacthen test results, the salivary steroid profiles were normal.

Eight patients had morning salivary cortisol concentrations below the 25^{th} quartile compared with the control population. All of these patients had a normal cortisol response either to insulin induced hypoglycaemia or to low dose synacthen. (See Table 6.12).

6.8.3 Abnormal

Two patients had both an abnormal response to low dose synacthen as well as an abnormal salivary steroid profile (G05 and B22). In one patient (G05) a urinary

metyrapone test was performed which was also abnormal, suggesting that adrenocortical insufficiency was as a result of ACTH deficiency. These results also supported the clinical suspicion that these patients were adrenocortically insufficient. Table 6.13 summarises the comparisons of the salivary profiles with the low dose ACTH stimulation test and insulin hypoglycaemia provocation in all the study patients.

	SALIVA					CAPILLARY BLOOD SPOT					Low dose ACTH			Insulin Induced Hypoglycaemia		
	Cortisol					Cortisol					Cortisol			Cortisol		
Patient Number	8a.m.	11a.m.	5p.m.	8p.m.	11p.m.	8a.m.	11a.m.	5p.m.	8p.m.	11p.m.	Age when tested (yrs)	Basal	Peak	Age when tested (yrs)	Basal	Peak
G06	7.4	14.2	6.5	8.9	1.9	130	303	112	131	53	14.5	682	780	5.5	500	750
G02	9.9	8.9	2.8	1.9	2.8	116	81	35	34	45		ND	ND	9.4	139	956
G03	8.8	4.8	4.2	2.3	1.7	144	66	36	11	9	12.2	256	604	9.4	292	887
G13	8.1	5.3	12.4	4.4	5.4	107	68	97	83	200	14.1	190	554	11.8	196	443
G10	7.7	5.7	3.8	3	3.6	138	69	57	18	19		ND	ND		?	?
G05	1.3	1	1	1	ND	ND	ND	ND	ND	ND	14	124	267	13.5	993	1472

	SALIVA					CAPILLARY BLOOD SPOT					Low dose ACTH			Insulin Induced Hypoglycaemia		
	Cortisol					Cortisol					Cortisol			Cortisol		
Patient Number	8a.m.	11p.m.	5p.m.	8p.m.	11p.m.	8a.m.	11a.m.	5p.m.	8p.m.	11p.m.	Age when tested (yrs)	Basal	Peak	Age when tested (yrs)	Basal	Peak
B18	9.3	5.4	3.6	2.3	1.8	259	82	20	10	30	14.3	349	650	12.3	407	690
B1	6	9.8	3.6	4	1.9	176	110	86	76	49		ND	ND	14.3	307	612
B7	9.2	4.9	6.2	3.6	2.6	138	76	85	39	30	19.4	493	1026	12.3	450	1029
B16	0.9	0.9	0.9	0.9	0.9	ND	ND	ND	ND	ND	17.7	27	58	13	173	537

(ND = No Data)

Table 6.12. Table showing all results of study patients with maximum morning salivary cortisol concentrations below the 25th Quartile.

Patient	Salivary Profiles	Low does ACTH test	Capillary Profiles
G01	N	N	N
G02	BN	-	N
G03	BN	N	N
G04	N	N	N
G05	A	A	-
G06	BN	N	N
G07	N	N	N
G08	N	N	N
G09	N	N	N
G10	BN	-	N
G11	N	N	N
G12	N	-	-
G13	BN	N	N
G14	N	N	N
G15	N	-	-
B01	BN	-	-
B02	N	-	N
B03	N	N	N
B04	N	-	N
B05	N	N	N
B06	N	N	N
B07	BN	N	N
B08	N	N	N
B09	N	-	N
B10	N	N	N
B11	N	N	N
B12	N	N	N
B13	N	N	N
B14	N	N	-
B15	-	N	-
B16	A	A	-
B17	N	N	N
B18	BN	N	N
B19	N	N	N
B20	N	N	N
B21	N	N	N

N = Normal A = Abnormal BN = Borderline normal

Table 6.13. Summary of Results in Study Patients

6.9 Summaries of Cases with Abnormal Profiles

6.9.1 Patient Summary 1: Christopher (B16)

Date of Birth 18/2/77

Christopher had failed a school eye test one year prior to presentation, and had been wearing spectacles. At the age of six, two weeks prior to admission he was noted to have right sided weakness, with a poor grip and a slight limp.

A CT scan showed an unusual, almost cystic lesion in the posterior part of the basal ganglia on the left, displacing the third ventricle. There was a soft tissue mass in the posterior part of the cyst, and a central increase in density in the suprasellar region extending across to behind the right carotid bifurcation.

Ophthalmic examination suggested a right homonymous superior quadrantic field defect and bilateral optic atrophy.

He was taken to theatre and a jelly-like tumour was found between the left side of the optic chiasm and the left carotid artery, extending back to involve the hypothalamic region. Biopsy showed this to be a low-grade astrocytoma (optic glioma). The cyst was drained and 20 ml of fluid removed.

Post-operatively he had diabetes insipidus, which subsequently settled after a short course of DDAVP. However, the DI recurred and he has required DDAVP since.

He was treated with radiotherapy in October 1983 (4500 cGy). An insulin hypoglycaemia test in November 1983 suggested he was growth hormone deficient, but was not initially commenced on GH therapy. GH was started in 1986.

He had a recurrence of the tumour cyst in May 1984, and an Ommaya reservoir was inserted to drain the cyst.

In 1989, thyroid function tests suggested hypothalamic hypothyroidism and was commenced on thyroxine replacement.

In February 1990, he was admitted with a history of increasing thirst and polyuria, feeling generally unwell with tiredness. A synacthen test suggested that he was cortisol deficient and was commenced on hydrocortisone replacement.

He was commenced on testosterone replacement in August 1990.

In July 1994, the decision was made to challenge the diagnosis of hypoadrenalism, and his steroid replacement was cautiously withdrawn over 12 to 14 weeks. A low dose synacthen test showed no cortisol response, and a urinary metyrapone test was abnormal. His salivary steroid profiles showed a flat cortisol profile, confirming ACTH deficiency. He was therefore recommenced on hydrocortisone.

He is currently doing very well, although is somewhat obese. He has reached a final height of 178.6 cm (above 50th centile). He has completed puberty although only has 2-3 ml testes in keeping with gonadotrophin deficiency.

6.9.2 Patient Summary 2: Mary (G05)

Date of Birth 10/03/80

Diagnosed aged 12 years following a six month history of intermittent headache, and a right convergent squint. Just prior to admission she developed a history of increasing lethargy, weight loss, and headaches.

On presentation, she had bilateral papilloedema. A CT scan showed an enhancing suprasellar mass.

A biopsy of the mass confirmed a germinoma. Bilateral VP shunts were inserted and she was treated with chemotherapy (combination of bleomycin, etoposide, and cisplatin in six courses). She was then given radiotherapy, 4000 cGy in 23 fractions over 33 days.

Post surgery there was evidence of secondary hypothyroidism (Total T4 49nmol/l, TSH 0.22 mU/l) and diabetes insipidus, and was commenced on thyroxine and DDAVP.

A repeat CT scan showed resolution of the tumour mass.

Following radiotherapy she had profound weight loss and required a short spell in hospital for nasogastric feeds.

Anterior pituitary function tests in August 1993 showed complete GH deficiency, gonadotrophin deficiency and TSH deficiency. The cortisol response was normal, with a peak of 1472 nmol/L.

She was commenced on GH therapy in addition to thyroxine and DDAVP.

Over the following six months she continued to have problems with weight loss and vomiting. In March 1994, salivary steroid profiles showed a flat cortisol profile, which was confirmed by a low dose synacthen test and urinary metyrapone test.

Once she was commenced on hydrocortisone replacement, she made a dramatic improvement, with weight gain, and improved appetite.

She was commenced on oestrogen replacement aged she was diagnosed as having partial gonadotrophin deficiency and was commenced on oestrogen replacement therapy.

To date, her main problems are of increasing weight gain, but this is partly due to overenthusiastic use of hydrocortisone during trivial illness.

At 17.5 years of age she developed classical symptoms of adult GH deficiency with extreme tiredness, lethargy, and reduced exercise tolerance. She was commenced on GH therapy. Her response to treatment was dramatic with resolution of her symptoms.

Repeat CT scans have shown no evidence of recurrence.

6.9.3 Patient Summary 3: Gareth (B18)

Date of Birth 30/11/78

Presented at 11 years of age with a five weeks history of persistent headache, nausea and dizziness. Clinically there was a left sixth nerve palsy, but no papilloedema at presentation.

A CAT scan showed a medulloblastoma, and a sub-total surgical removal was performed (95% removal) together with pre-operative shunting, followed by radiotherapy. He received 3500 cGy in 21 fractions over 29 days to the whole brain and spinal axis, and 1980 cGy in 11 fractions over 17 days to the tumour site, along with dexamethasone.

He did very well following his radiotherapy.

Aged 12 years, evaluation of his anterior pituitary function was performed. This showed growth hormone deficiency, but no other endocrine deficit. He was commenced on growth hormone therapy, with good effect.

At the age of 14 he began to develop symptoms that may have suggested ACTH deficiency, with tiredness, lethargy, lacking in energy, and prolonged recovery following viral illness. A low dose synacthen test showed a normal cortisol response with a peak cortisol concentration of 650 nmol/L. However, salivary steroid profiles showed loss of diurnal rhythm, with morning cortisol concentrations at the lower limit of normal. He was commenced on hydrocortisone replacement with significant positive clinical response with long lasting improvement in his symptoms. He continued to do well, with a good quality of life.

At the age of 17.5 years, he had reached a final height of 170.2 cm, and had completed secondary sexual development.

At the age of 20 years, he continues to do well with no problems to date. He continues on hydrocortisone replacement therapy with no adverse effects. He is now regularly reviewed by the adult oncologists and endocrinologists.

6.9.4 Patient Summary 4: Kelly Ann (G10)

Date of Birth 5/2/1980

Presented at the age of 3 years with a divergent squint in the right eye. Surgery to correct the squint was performed in 1983 aged 13 years, but the squint gradually recurred with poor vision in the right eye. Initially, fundoscopic examination was normal. Subsequently, in early 1984, she was noted to have right optic atrophy. A skull film showed an enlarged pituitary fossa with erosion of the anterior clinoid processes and thinning of the dorsum sellae, with widening of the right optic canal.

A CT scan showed widening of the right optic nerve infra-orbitally, with evidence of a suprasellar mass extending posteriorly and to the right.

At craniotomy, the optic nerves and chiasm were found to be involved in a large mass of solid tumour. There was no hypothalamo-pituitary involvement. Biopsy of the lesion confirmed an optic nerve glioma.

She was treated with radiotherapy in April 1984, with a total dose of 5000cGy in 30 fractions over 44 days, using a three field plan and 4 MeV x-rays. The treatment was effective in shrinking the tumour. She was subsequently diagnosed as having neurofibromatosis with multiple café au lait spots.

Anterior pituitary function testing was performed in 1986, aged 6 years, which demonstrated growth hormone deficiency. She was started on growth hormone therapy with good results.

Aged 8 years, she showed pubertal progression, with biochemical and bone age evidence of precocious puberty, and was commenced on Buserelin (an LHRH analogue). She showed little pubertal progression on this treatment. In 1990, aged 10 years, a TRH test also showed evidence of primary hypothyroidism and was commenced on thyroxine replacement.

In 1993, aged 13 years, Kelly Ann developed symptoms of tiredness, especially early morning, and difficulty in shaking off trivial infection.

A salivary steroid profile showed subnormal cortisol concentrations, suggestive of hypoadrenalism, and she was commenced on hydrocortisone replacement. She had a good clinical response to replacement therapy that has continued.

At the age of 16 years, she had reached a height of 156.8 cm, was pubertal (pubertal stage B5) and menarchal, and continues on thyroid and hydrocortisone replacement.

7 Discussion

The accurate diagnosis of secondary adrenocortical insufficiency is a great challenge, particularly in the childhood population of cancer survivors. The diagnosis is difficult to make with confidence, as standard tests of adrenal function can give misleading results (Borst, Michenfelder, & O'Brian 1982). There continues to be a lively debate as to which test is the best and most accurate, (Clayton 1997) with convincing arguments for whichever test is favoured. The debate also continues regarding the accuracy of tests of adrenal reserve, and their significance in everyday life in terms of the individual's ability to cope under physiologically stressful events. It is arguable that the assessment of adrenal steroids in normal physiological conditions is at least as accurate in determining adrenal function as dynamic tests. Adrenal steroid status is a dynamic process and the value of non-physiological stimuli to assess adrenal status remains controversial.

The use of saliva in the assessment of hypothalamo-pituitary-adrenal function is attractive; it is non-invasive, stress-free and requires little if any supervision.

One aim of this study was to develop and evaluate sensitive assays of adrenal steroids in saliva to allow measurement of salivary steroid day profiles in patients at risk of developing adrenocortical. With advances in RIA techniques and with the development of specific antibodies, accurate measurement of these steroids in saliva was possible. Adrenal steroid immunoassays have been developed which are extremely sensitive, even using small sample volumes. The salivary cortisol assay developed as part of this study was developed specifically for use in screening children at risk of developing SAI. As a consequence, there were two essential requirements:

1. The assay had to be as sensitive as possible in order to be able to detect very low concentrations of cortisol in saliva
2. The assay could be performed with very small sample volumes (50μl), because of the potential difficulties in obtaining samples of saliva in children.

Read, Riad-Fahmy, Walker, et al, at the Tenovus Institute in Cardiff (Riad-Fahmy, Read, Walker, & Griffiths 1982) performed much of the foundational work on salivary steroid profiles. There is now a great deal of research evidence on the use of saliva and the behaviour of a variety of steroid hormones in saliva is now accurately described. It was on this foundation that the salivary steroid assays for this study were developed.

7.1 Salivary steroid assay development

The assays developed in this study were found to be robust and gave reliable and reproducible results. The assays developed were also relatively simple and quick to perform. Unfortunately, there is little comparative data with which to compare the results of these assays with other studies.

7.1.1 Salivary Cortisol Assay

A simple non-extraction assay using uncomplicated and well-established routine laboratory techniques was developed. The use of encapsulated antibody made the assay more robust as the antibody-ligand complex was more stable and separation of antibody-ligand complex from ligand was simple to perform (Wallace 1992). The use of iodinated label was also advantageous because it had a high specific activity, and was easy and relatively cheap to detect, despite its relatively short half-life (Gilby et al 1973; Lindberg & Edqvist 1974). The assay was technically easy to

perform, allowed easy processing of large numbers of samples and produced reproducible results in a relatively short time.

An assay sensitivity of 0.9 nmol/L suggested this was a highly sensitive assay. Moreover, it had acceptable intra- and interassay coefficients of variation, low cross-reactivity, and hence high specificity.

7.1.2 Salivary 17-Hydroxyprogesterone and androstenedione assays

The 17-OHP and androstenedione assays were adapted from previously developed assays in the regional endocrine laboratory in Glasgow.

The 17-OHP assay developed in this study similarly made use of encapsulated antibody; the method was very similar to the salivary cortisol assay. The sensitivity and precision of the assay were adequate to the task.

The androstenedione assay was more laborious and time consuming, involving solvent extraction prior to radioimmunoassay. The solvent extraction process was necessary because of the presence in patients' samples of high concentrations of cross-reacting polar conjugates of steroids (Goodall & James 1981). The presence of steroid binding proteins as a possible source of interference was not relevant in this case, as only very small amounts of these are present in saliva and so are not significant. The principal binding protein appears to be SHBG, but is present in saliva in very low concentrations (Selby, Lobb, & Jeffcoate 1988).

The assay sensitivity was again excellent (0.35nmol/L).

7.1.3 Capillary Blood Spot Steroid Assays

Apart from the blood spot cortisol assay, the capillary blood spot assays for 17-OHP and androstenedione were developed in-house. Details of their development have been published elsewhere (Chearskull 1985; Santiago et al 1996).

7.1.4 Capillary Blood Spot Cortisol Assay

The main problem with measuring cortisol in blood spots was in overcoming the effects of protein binding that affected antibody-ligand binding. Initial problems with the blood spot cortisol assay were resolved once incubation times were greatly increased and the assay buffer was changed to citrate/phosphate (pH 4.0). This was found to be superior to ANS as it did not interfere with antibody binding but was effective enough to inhibit protein binding, giving optimal assay sensitivity. The assay had a sensitivity of 20nmol/L whole blood (equivalent to 40nmol/L serum) and a CV of <10% throughout the working range.

It was unfortunate that control data could not be obtained using capillary blood spot samples as this was not approved by the local ethics committee.

However, valuable information was gained with the study patient samples that could pave the way for development of this aspect of monitoring in the future.

7.2 Control Population Results

The results of the salivary profiles from the control population demonstrated the feasibility of the assay and collecting domiciliary salivary samples in children. Although there was no way of assessing whether the instructions were adhered to, the fact that only 12.5% (21 out of 168 profiles) of the profiles could not be used was probably a reflection on the simplicity of the technique of saliva collection. These profiles were excluded from the study because they were of poor sample quality or low sample volume. All of the remaining profiles showed a diurnal variation of all three steroid hormones measured. This suggested that the use of a simple method of obtaining a steroid profile was possible in a home setting. Arguably, obtaining steroid profiles in a relatively stress-free environment ensures that the profile is an accurate assessment of physiological adrenocortical function.

It is unfortunate that none of the control subjects provided samples of saliva when they were unwell.

7.2.1 Salivary Cortisol Profiles

The salivary cortisol profiles exhibited a diurnal pattern with peak salivary cortisol concentrations in the morning. There was acceptable intra-individual variability in the morning samples; a maximal value was obtained in at least one of the two morning samples, but many of the profiles had peak concentrations in both morning samples.

When comparing the consecutive morning samples in the 139 controls that provided two samples, there is a similar spread of results in both groups. There is however a relatively low correlation coefficient, suggesting perhaps more variability than might be expected. It has been suggested that a peak cortisol concentration may be missed if a specific time of the day is chosen (Katz & Shannon 1964). The reason

that some of the profiles did not show peak concentrations in both morning samples might be that the samples were not taken at the same time each day in each individual. The controls were asked to take the morning sample at 8 a.m. because it was felt that this was the most convenient time, and because this was most likely to be around the time of wakening. Peak cortisol concentrations have been shown to be related more to the time of wakening; cortisol concentrations begin to fall within 30 minutes of wakening (Lindholm et al 1978). The results would therefore suggest that several morning samples should be obtained to ensure that maximal salivary cortisol concentrations are obtained.

There was no correlation with salivary cortisol and age and body size, as has been noted in other studies looking at both serum and salivary cortisol concentrations. The fact that the salivary cortisol reference range in the control children studied is similar to adult reference ranges in other studies also suggests that age does not affect cortisol concentrations. The values obtained in the control population compare favourably with control values in other studies (see Table 7.1). There was a wide range of cortisol concentrations in the controls, particularly in the first morning samples, ranging from 4.6nmol/L to 35.9nmol/L. The salivary cortisol concentration in the morning sample may also reflect what the child was doing at that time of day, as well as indicating true physiological basal concentrations. In some of the children, the 8a.m sample may not have been taken at the time of that particular child's peak cortisol production and hence may not demonstrate the child's maximal cortisol concentration if the time for maximal cortisol production was missed. This would then result in a misleading result and lead to an erroneous suspicion of SAI in the study patients. The samples were also not necessarily taken when the child was at rest. For example, if the child had run up and down the stairs prior to obtaining a sample of saliva, then this may result in an inadvertently high salivary cortisol

concentration. This would not be a problem when looking for evidence of adrenocortical insufficiency, but would be a problem when determining cortisol excess.

Thus when identifying SAI only the first morning sample is informative. This is further suggested by the finding that calculation of AUC gave no further information.

If one hypothesised that in hypoadrenalism, total cortisol production is reduced, then calculation of the area under the curve might provide an estimate the total daytime cortisol release. Calculation of the area under the curve for salivary cortisol unfortunately did not provide any additional information regarding adrenal function. Calculating the area under the curve was simply an alternative method for obtaining the average concentration of salivary cortisol throughout the day, with the effect of diminishing the importance of the morning cortisol concentration.

The lack of a significant sex difference for salivary cortisol concentration was also in keeping with several other studies (See Table 7.1 comparing cortisol results from various studies).

Study authors	Subjects	Age (years)	Morning salivary cortisol	Evening salivary cortisol
Al-Ansari et al 1982	Men (n=62) Women (n=88) Children (n=105)	? ? ?	12.0 10.0 11.0	
Hiramatsu 1981	Men (n=8) Children (n=8)	24 - 33 ?	9.7 8.3	2.8 1.4
Laudat et al 1988	Men (n=23) Women (n=35)	24 - 50 24 - 50	16.2 9.8	3.9 3.9
Umeda et al 1981	Men (n=10)	24 - 33	27.3	14.0
Vining et al 1983	Men (n=5) Women (n=5)	24 - 32 24 - 32	25.5 21.0	3.4 2.7
Kirschbaum & Hellhammer 1989	Men and Women	?	14.3	1.9
Current Study	Children (n=147)	5 - 15	14.5	1.4

Table 7.1. Comparison of Salivary Cortisol Concentrations from Various Studies.

7.2.2 Salivary 17-Hydroxyprogesterone Profiles

A diurnal pattern of 17-OHP concentration was also found in the salivary steroid profiles. In general, the daytime 17-OHP concentrations were very low, with a large proportion of the daytime samples having salivary 17-OHP concentrations below the limit of sensitivity of the assay. Of the daytime salivary 17-OHP concentrations, 107 out of 141 samples (76%) were below the limits of sensitivity of the assay.

The sex difference found with the maximum and minimum salivary 17-OHP ratios was small but statistically significant. Salivary 17-OHP concentrations were slightly higher in adolescence in girls than in boys. The correlation between age and 17-OHP in girls may be due to a variety of reasons. 17-OHP is produced in the ovary, testis and the adrenal gland. In older children the proportion of 17-OHP produced by the gonads becomes more significant as children progress through puberty. Increased 17-OHP concentrations are also found in adrenarche (Tanner & Gupta 1968).

Maximum 17-OHP was not shown to increase with age in boys, probably because sex steroid concentrations in boys rise predominantly in the second half of puberty, while our sample population of boys were either prepubertal or in early puberty.

The fact that minimum salivary 17-OHP concentrations also rise in relation to age and body surface area, might also be further evidence to suggest that the gonadal component of salivary 17-OHP production becomes more significant with age and with the onset of puberty. One must presume that a significantly larger proportion of the older girls in the control group were in established or late puberty. It is known from previous studies in children that activation of the adrenal cortex occurring in adrenarche and early puberty occurs later in boys than in girls (Tanner & Gupta 1968). If salivary profiles were obtained in boys up to the age of approximately 17

years, then perhaps we would see a correlation in salivary 17-OHP concentrations with age and body size. This would be an area for future study.

7.2.3 Salivary Androstenedione Profiles

The androstenedione concentrations in the salivary profiles followed a similar diurnal pattern to that obtained for salivary cortisol (See Table 4.5).

The salivary androstenedione concentrations were also found to correlate positively with age in girls at both maximum and minimum salivary androstenedione concentrations. Area under the curve also correlated positively with age. As with salivary 17-OHP profiles in this study, there was no such correlation found with the male control group, presumably for the same reasons as with salivary 17-OHP.

The sex difference noted for both maximum salivary androstenedione concentrations and also AUC has been described previously with serum concentrations but not in saliva (Apter et al 1979). Salivary androstenedione concentrations were higher in all age groups tested.

Little work has been published with regard to salivary androstenedione concentrations in normal children. In the study by Young (Young, Walker, Riad-Fahmy, & Hughes 1988b), the increase in salivary androstenedione with age is more noticeable than with 17-OHP concentration. Presumably with advancing age, either the gonadal production of androstenedione assumes greater importance, or there is predominantly greater androstenedione production in the adrenal gland in males than in females. However, adrenal androgens have been shown to rise during childhood, at least in part due to adrenal maturation (Tanner & Gupta 1968).

7.2.4 Salivary Steroid Hormone Ratios

The reason for calculating salivary steroid ratios was to determine whether changes would occur in the ratios of adrenal steroids with diminishing ACTH drive, and if this would be helpful in the diagnosis of SAI.

The difference in ratios at maximum and minimum salivary cortisol concentrations is presumably a consequence of a greater change in salivary cortisol through the day than salivary 17-OHP; salivary cortisol concentrations have a proportionately greater fall than salivary 17-OHP. There was no sex difference for ratios at both maximum and minimum salivary cortisol concentrations. The spread of results was greater in boys than girls.

The fall in cortisol/17-OHP ratios with age in boys is difficult to explain, as no correlation was found for the cortisol and 17-OHP concentrations. However, the correlation is just at the level of significance and this relationship may be coincidental.

There is a slight fall in salivary cortisol/androstenedione ratios from maximum to minimum salivary cortisol concentrations. This again may be explained because of a greater fall in salivary cortisol than salivary androstenedione.

There is no sex difference found with these ratios, presumably because the relative ratios between the two steroids are the same.

The ratios at maximum salivary cortisol concentrations are lower than at minimum concentrations, probably as a result of a greater fall in salivary 17-OHP than androstenedione. Once more there is no sex difference for the ratios at maximum and minimum salivary cortisol concentrations.

7.3 Study Group Results

The steroid profiles performed in the study group were performed under similar instructions as with the control group, and at the same times throughout the day. In this way, a direct comparison could be made between the study patients and controls. Both saliva and capillary blood samples were taken in 31 study patients (13 girls), enabling the relationship between saliva and capillary blood spot profiles to be studied. In addition, some of the study patients provided profiles when they were unwell as well as when they were well. Although these profiles could not be directly compared with the control group (as none of the control group provided unwell profiles), the well and unwell profiles for each patient could be compared.

7.3.1 Salivary cortisol profiles

The salivary cortisol profiles in the study patients were comparable with the control group except in two patients, who did not demonstrate a diurnal pattern of variation in cortisol concentration. These patients had salivary cortisol concentrations at the limit of sensitivity of the salivary cortisol assay. One of the patients (G05) had symptoms suggestive of hypoadrenalism. In a low dose Synacthen test the patient failed to demonstrate a cortisol response to ACTH, and the patient also failed to show increased 11-hydroxysteroid concentrations with an oral metyrapone challenge. In the other patient (B22) there was an initial diagnosis of SAI made on clinical grounds. However, this diagnosis was in doubt, and with no good objective evidence of hypoadrenalism, he was tentatively weaned off steroid replacement therapy over eight weeks, following which salivary profiles were performed over two days. These profiles failed to show any measurable cortisol in saliva. A low dose Synacthen test also failed to demonstrate a cortisol response to ACTH, and he had an absent response to metyrapone in a three day urinary metyrapone test.

Two other patients with symptoms suggestive of hypoadrenalism, had profiles that were at the lower end of the normal range, although were not frankly abnormal. In one of these patients (B18), a low dose Synacthen test was normal. He was commenced on hydrocortisone replacement following which his symptoms improved. He has continued to remain symptomatically well. The other patient (G10) was found to have a normal Synacthen test and a salivary profile at the lower end of the normal range. This patient was commenced on hydrocortisone but this was later discontinued. The symptoms subsequently recurred and hydrocortisone was recommenced with good effect.

In patients who are developing SAI there may be subtle changes in their salivary cortisol profiles, and their salivary cortisol concentrations may be at the lower end of the normal range. However, in the patients in this sub group (see Table 6.12), other tests of adrenal function failed to show any evidence of hypoadrenalism. It is important that this group of patients are followed to determine whether they may develop SAI in time.

As expected, comparison of the well and unwell profiles for each of the remainder of the patients in general showed an increase in salivary cortisol concentrations in response to stress, which was usually an acute viral illness. However, the difference in salivary cortisol concentrations was variable. This is most likely to be because of the subjective nature of the illness and the patient's own interpretation of being unwell. Although the patients were given a strict definition of what was to be accepted as being unwell, the final interpretation of the definition was dependent on the patient and his/her family's perceptions. It could not be concluded from the current study whether any of the study patients had abnormal steroid profiles in response to stress. In those patients with significantly raised salivary cortisol

concentrations in response to stress, the salivary cortisol concentrations were raised throughout the day, and not just in the morning samples.

In patients at risk of developing SAI, perhaps when looking for subtle changes in adrenal function there may be an initial loss in the daytime increase in the cortisol response to stress. This would need significantly more data to show whether this was indeed the case, and also more control data in stress situations.

7.3.2 Salivary 17-Hydroxyprogesterone and Androstenedione Profiles

The salivary 17-OHP profiles were also comparable with those of the control group. The concentrations in the patients with flat salivary cortisol profiles were also comparable, with no reduction in 17-OHP concentrations in the morning. This is presumably because the salivary 17-OHP concentrations are already low and so it would be very difficult to detect even lower concentrations.

When the two groups were compared (excluding the patients with definitely abnormal profiles), there was no statistically significant difference between them. However, the median maximum and minimum salivary 17-OHP concentrations were slightly higher in the male study group than in the male control group, but slightly lower in female study group than in the female control group.

In the patients with abnormally low salivary cortisol profiles, the 17-OHP profiles were not of any additional help. In fact, they were somewhat misleading, as the 17-OHP concentrations were generally within the reference range. This again supports the hypothesis that the 17-OHP levels are simply reflecting gonadal function rather than adrenocortical steroid release.

The salivary cortisol/17-OHP ratios for maximum and minimum salivary cortisol concentrations followed the same pattern as with the control group.
However, interestingly, the stressed ratios were higher than the basal

concentrations at maximum salivary cortisol concentrations, but the same when the basal and stressed ratios at minimum salivary cortisol were compared. This suggests that there is a significantly greater relative fall in salivary 17-OHP concentration under stress compared with the increase in cortisol concentration under stress, implying that the cortisol synthetic pathway is driven towards cortisol synthesis in stressful situations. Thus, it is possible that an increase in ACTH drive results in an acceleration of the adrenal steroid biosynthetic pathway, such that there is preferential cortisol (and possibly mineralocorticoid) production at the expense of androgenic steroid production, resulting in relatively lower concentrations of the steroid precursors such as 17-OHP.

The ratios of salivary cortisol and androstenedione followed a similar pattern to the salivary cortisol/17-OHP ratios. The ratios were higher under stress compared with basal conditions at maximum salivary cortisol concentration. However, the ratios at minimum salivary cortisol concentration were higher under stress than under basal conditions. This suggests that cortisol is preferentially synthesised at the expense of androstenedione in times of stress. Thus, the steroid pathway may again be diverted along the cortisol biosynthetic pathway, with relatively less androgens being produced. A further study is required to confirm this.

The salivary 17-OHP/Androstenedione ratios increase with stress at maximum salivary cortisol concentrations but not at minimum concentrations. The change in ratios is not as striking as with the ratios involving cortisol. However, there is a definite rise in 17-OHP/androstenedione ratio at maximum salivary cortisol concentrations. This suggests that there is a relative increase in 17-OHP concentration compared with androstenedione concentration resulting from either a proportional increase in 17-OHP or a decrease in androstenedione. If this is the case, then it may be that cortisol production is increased at the expense of

androgen production, as well as increasing cortisol production by utilising 17-OHP in the cortisol biosynthetic pathway. It might be that this situation also occurs in conditions of falling ACTH concentrations, when enzyme sensitivity to ACTH may change in favour of cortisol synthesis. This is speculative, as unfortunately, there were insufficient numbers of patients with hypoadrenalism to confirm this. Further studies may provide further information as to whether this hypothesis is correct.

7.3.3 Capillary Blood Spot Steroid Profiles

The capillary blood spot profiles performed in the study patients similarly followed a diurnal pattern, with close correlation with the salivary profiles. This correlation was quite variable in the study patients, with some profiles showing poor correlation with the salivary profiles taken at the same time. This might reflect the inadequacy of obtaining satisfactory blood spot samples. In a significant number of study patients, the quality of blood spot samples obtained was poor. It is also possible that some of the blood spot samples were not taken at the same time as the saliva samples and so a correlation could not be obtained. However, in reliable patients in whom it was felt that the instructions regarding taking samples was closely followed, there was excellent correlation between saliva and capillary profiles, with some patients exhibiting a correlation coefficient between saliva and capillary blood spot profiles of 0.9 or greater.

As there were no capillary blood spot samples in the control group, no comparison with normal children could be made.

7.3.4 Low dose Synacthen Test

The low dose Synacthen test has been introduced over the past ten years. It is relatively straightforward to perform, and it has also been shown to give results comparable to the standard short synacthen test when compared with healthy

adults. It has also been shown to give results equivalent to a physiological response to ACTH in healthy adult subjects (Crowley, Hindmarsh, Holownia, Honour, & Brook 1991).

In this study, the results of the low dose synacthen test confirmed the presence of hypoadrenalism in the two patients with abnormal profiles.

The results showed that using all the criteria for the standard 250μg synacthen test resulted in a false positive rate of 29%. However, only the patients with abnormal salivary profiles failed to reach a peak serum cortisol concentration of at least 500 nmol/L. If this criterion is used in diagnosing SAI, then the two patients with borderline low profiles would not be diagnosed if only the low dose synacthen test is used, as these patients had a normal response to synacthen. It is significant to note that an abnormal response to low dose Synacthen was not always associated with an abnormally low salivary cortisol concentration. Conversely, an abnormal salivary cortisol profile was always associated with an abnormal response to low dose Synacthen.

The low dose Synacthen test has been heralded as a sensitive method of assessing physiological adrenocortical reserve, and as such is felt to be a useful test in diagnosing adrenocortical insufficiency, including SAI. However, in this study, we have shown that the results of the low dose Synacthen test may be misleading, and may result in an over diagnosis of SAI, and result in inappropriate treatment of these patients with hydrocortisone replacement. Previous studies have also highlighted problems in the interpretation of the results of tests of adrenal reserve and have also shown discrepancies between the different tests performed (Borst, Michenfelder, & O'Brian 1982; Cunningham, Moore, & McKenna 1983; Tsatsoulis, Shalet, Harrison, Ratcliffe, Beardwell, & Robinson 1988).

The results of the low dose Synacthen test in this group of patients illustrate the problems associated with using the synacthen test in diagnosing hypoadrenalism secondary to ACTH or CRH deficiency, and it is therefore not recommended as a method to be used alone in the diagnosis of SAI.

7.3.5 Single dose oral three day urinary metyrapone test

A three day urinary metyrapone test was performed in five patients in this study. This was primarily to assess the efficacy of the test, and the practicalities of performing the test as a procedure that could be performed at home. It was performed in two patients (M.W; C.M.) who had an abnormal salivary cortisol profile and an abnormal low dose Synacthen test.

The administration of even a single relatively small dose of metyrapone in these patients resulted in an accentuation of symptoms that were more attributable to hypocortisolism. Although the results of the metyrapone test were abnormal, the results were relatively subtle. It is questionable as to whether it is appropriate to subject patients to metyrapone who are suspected of being cortisol deficient, and so exposing them to even greater risks of adrenal crisis in a situation where there may not be immediate assistance.

As a screening test in its own right, it probably does not have anything additional to offer over the salivary profiles with regard to ability to screen for SAI.

7.3.6 Insulin hypoglycaemia provocation

None of the study group had an ITT performed if the diagnosis of SAI was suspected. The majority of patients in the study had had an ITT up to a year previously, and further exposure to unnecessary risk of the procedure was felt to be unethical and inappropriate. Other studies have suggested that patients with SAI may have an abnormal cortisol response to hypoglycaemia, but a normal Synacthen

response (Borst, Michenfelder, & O'Brian 1982; Cunningham, Moore, & McKenna 1983) and vice versa (Tsatsoulis, Shalet, Harrison, Ratcliffe, Beardwell, & Robinson 1988). This begs the question as to which of the tests of adrenal reserve is actually demonstrating an abnormality, and which is showing an inappropriately high false positive rate.

8 Conclusions and Recommendations

This study highlights the difficulties in diagnosing SAI in this particularly complicated group of patients. The need for continuing screening for SAI is paramount, as these patients are at risk of developing SAI following treatment. The patients are also at potential risk of having many years of ill health and morbidity as a result of SAI which may not be detected, and which may be partial and yet which can still produce vague symptoms of ill health.

The use of salivary profiles is a simple, sensitive procedure for assessing adrenal function in a physiological way and can be used to monitor adrenal function in these children. This avoids repeated provocation tests to assess adrenal function. Saliva collection is generally well tolerated by the children studied as well as by the control group. As a group, the survivors of childhood cancer will require life-long surveillance of their endocrine status. This is true not only of adrenal function, but also of thyroid function. The use of saliva in monitoring adrenal function is a safe, non-invasive method that can be used as often as is necessary.

This study has shown that the results of the peak morning salivary cortisol concentrations are probably as informative as day profiles. We therefore recommend that morning and evening samples should be collected on three consecutive days. If the results are subnormal (the peak salivary cortisol concentration is less than 6.5nmol/L), a low dose synacthen test should be performed. If the results of the low dose Synacthen test are equivocal or normal, then the salivary samples should be repeated, or if there are symptoms highly suggestive of hypoadrenalism, then one could tentatively commence steroid replacement and critically assess the response to treatment.

In patients in whom the salivary profile remains consistently at the lower end of the normal range (below the 25th quartile of the reference range, i.e. a cortisol concentration of less than 10.3 nmol/L), the salivary profile should be repeated. If the repeat profile shows a persistently low cortisol in the morning (less than 10.3 nmol/L), low dose synacthen test should be performed in order to ascertain their degree of adrenal reserve. The question is whether these patients who have low normal salivary cortisol profiles but a subnormal response to low dose synacthen should be given hydrocortisone in times of physiological stress.

The salivary profiles should be repeated once a year if the patient remains well, and they should be performed more frequently if there are developing symptoms suggestive of hypoadrenalism.

Figure 8.1 is an algorithm demonstrating the management of children at risk or suspected of having SAI.

The use of an ITT in the assessment of adrenal reserve alone is not justified unless the growth axis is also to be assessed.

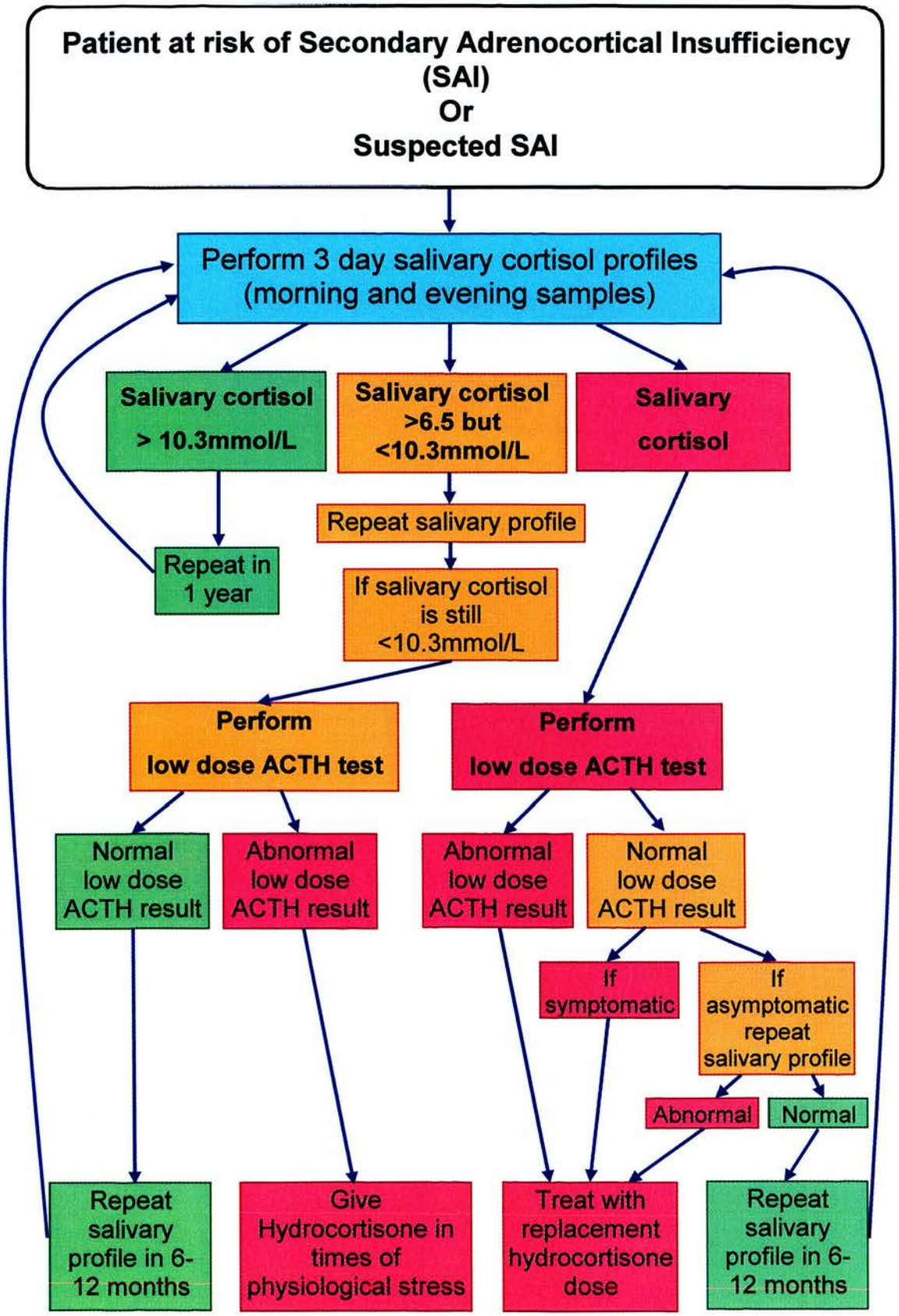

Figure 8.1. Algorithm for the Management of Children at risk of Secondary Adrenocortical Insufficiency

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10 Appendix

Appendix 1. Consent form for control group.

I do / do not* give permission to allow my child

_____, to provide samples of saliva **only** for a study being undertaken by Dr. John Schulga at the Royal Hospital for Sick Children, Yorkhill, for the measurement of steroid hormones.

I understand the nature of the study as explained to me in the information sheet provided by the research team at the hospital.

I also understand that the results of the tests will be strictly confidential.

I confirm that my child :-

1. Is not currently taking steroids in any form.
2. Has not been taking steroids in the past 2 weeks.
3. Does not have a long-term illness as far as I am aware.

Dr. John Schulga

Research Fellow in Paediatric Endocrinology.

* Please delete as appropriate.

Signature of parent/guardian*

_____

Signature of witness _____

Appendix 2. Information Sheet for Saliva Study.

INFORMATION SHEET FOR SALIVA STUDY

I am currently developing a test for measuring steroid hormones in saliva. This will replace blood tests, which can be painful and unpleasant, and at times difficult to perform in small children.
In particular I am measuring a steroid hormone in saliva called cortisol. This is produced naturally in the body, and is essential when you are unwell, for example, in helping to fight infections.
The reason for developing the test is so that we can diagnose children lacking these hormones. A particular group of children at risk of becoming deficient in cortisol are those who have been treated for various childhood cancers, including leukaemia.
With this saliva test, we will be able to let the children take samples at home, instead of having a blood test in hospital.

However, we need samples of saliva from normal healthy children, so that we know what the normal levels of these steroid hormones should be.

I would be most grateful if you would consider allowing your child to provide samples of saliva. There would be **NO** blood tests involved. I must also stress that **the results would be strictly confidential**.

Your child would be asked to provide 5 or 6 samples of saliva per day for 1 or 2 days; one day when they are well, and if possible, one day when they are unwell with a cough or cold and a temperature. This is done by simply spitting into a plastic tube.
Your child will also be weighed, and their height measured so that the results of the tests can be accurately compared.
The samples need to be **frozen as soon as possible after they have been taken**, so if you do not have ready access to a freezer, I am afraid you will not be able to do the test.
Also, if your child is currently taking steroids, for example, by inhaler for asthma (such as becotide, or pulmicort), then they will also be unable to take part, as these may interfere with the test.

If, after careful consideration, you feel that you can allow your child to take part, you will receive further information regarding the test in the near future. Approval for the study has been granted by the Department of Education at both regional and divisional levels.

Please complete the attached form, which is a requirement of the Hospital ethics committee, and return it to the class teacher at school. Please note that your signature needs to be witnessed. This can be done by anyone, including your husband or wife.

Thank you for taking time to read this letter.
Yours faithfully,
John Schulga
Research fellow in Paediatric Endocrinology.

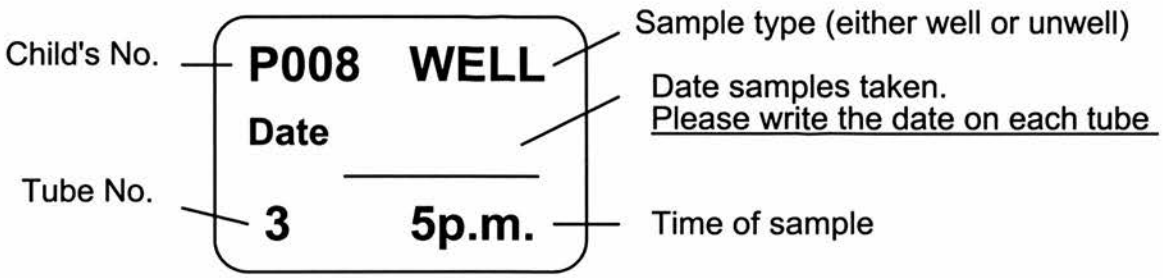
Appendix 3. Collecting Saliva Samples.

COLLECTING SALIVA SAMPLES

Thank you for agreeing to provide samples of saliva for this study.
The bottles are labelled 1 to 6 and also have a time on them. Please keep to the times as closely as possible. The labels are as follows:

1 - 8a.m. (08.00)
2 - 11a.m. (11.00)
3 - 5p.m. (17.00)
4 - 8p.m. (20.00)
5 - 11p.m. (23.00)
6 - 8a.m. (08.00 the following day).

The labels on each tube looks like this:



Please note that **there are two 8a.m. samples**.
If possible, please collect the 11p.m. sample, but if you feel that this is too late for your child, then ignore it.

Please collect the 6 samples over 24 hours, starting at 8a.m and finishing at 8a.m the next day.

There are two sets of bottles labelled **WELL** and **UNWELL**. Please collect samples in the WELL bottles when your child is his/her normal self.
It will be helpful if the samples were taken during the weekend beginning **Saturday 23rd, April,1994**, and brought to school on **Monday morning, 25th, April**.
Please remember to **FREEZE** the samples as soon as possible after they are taken.
IF POSSIBLE collect samples in the **UNWELL** bottles if your child has any of the following:

1. A temperature
2. A cough or cold that keeps them off school.

If your child is not unwell by the **20th, June, 1994**, then it is not necessary to collect **UNWELL** samples.

Technique for collecting saliva

1. **Do not** take samples for up to **one hour** after taking food or brushing your teeth.(E.g. take the morning sample before breakfast, and the 5p.m. sample before tea).

2. Rinse your mouth out with water for 20 or 30 seconds and empty your mouth.

3. Spit directly into the tube and fill the tube up to the mark on the tube (labelled 2.5ml). This may take a few minutes and several "spits".

4. Close the tube tightly and place the tube into the plastic bag provided and place the bag into the **freezer**.

5. Repeat this for each sample, putting the tube into the same bag each time.

6. Once all 6 samples have been collected and frozen, take them to school as soon as possible and give them to your teacher.

Dr. John Schulga, Research Fellow in Paediatric Endocrinology,
R.H.S.C., Yorkhill, Glasgow.

Appendix 4. Protocol for Capillary Blood Spot Testing

1. Please take finger prick blood samples on the following times:
    - 8.00a.m.
    - 11.00a.m.
    - 5.00p.m.
    - 8.00p.m.
    - 11.00p.m.
    - 8.00a.m. next day.
2. Wash hands in warm water a few minutes before finger pricking since warmth increases blood flow to the skin. Dry thoroughly since any dampness on the skin may affect the blood results.
3. Some children find that a prick on the side of the finger is less painful than on the finger pulp.
4. After pricking the finger don't squeeze it immediately but wait 10 seconds or so. Then squeeze from the base of the finger towards the prick - don't squeeze around the prick itself since this can actually stop the blood coming out!
5. One good drop of blood is usually enough to fill one circle.
6. Ensure that the blood soaks right through the paper to the other side and is not just smeared on one side of the paper.
7. Soak the blood on one side of the paper only (not a bit on one side and then a bit more on the other!).
8. Don't add extra blood to the spots after it has dried to make it bigger - if possible try to get one large drop from the finger before putting it on the paper so the spot does not have to be added to at all.
9. One circle filled with blood is sufficient to do the test, but if you can fill the second circle from the same finger prick this will help the laboratory.
10. Before putting the filter paper back in the envelope, let the blood dry. Once this has happened the blood spots won't "go off" for weeks provided that the strip is kept out of direct sunlight, in a dry place and at ordinary room temperature.

Appendix 5. Protocol for Low dose Synacthen Test

PROTOCOL FOR LOW DOSE SYNACTHEN TEST

1. Patient must be fasted overnight.

2. Calculate the dose of Synacthen to be given :

   Give 500 ng ACTH / 1.73 m^2 i.v.

3. To calculate the volume of synacthen to give :

   250 μg ACTH in 1 ml.

   Dilute to 10 ml with saline = 25 μg in 1 ml.

   Dilute 1 ml of this solution to 5 ml = 5000 ng in 1 ml.

   Dilute 1 ml of this solution to 10 ml = 500 ng in 1 ml.

   E.g. If the patient has a body surface area of 1.73m^2, the volume of solution to give will be 1ml i.v of this solution.

4. Take a basal venous blood sample (2ml volume) - time 0.

   Give ACTH as calculated.

   Take further blood samples at the following times - 10 min.

   15 min.

   20 min.

   25 min.

   30 min.

   35 min.

(Peak rise in cortisol occurs at around 15 mins. in 80% of patients)

c.f. S. Crowley et al.
J. Endocrinol. 1991; 130: 475 - 479.

Appendix 6. Daily Activities Record

Name : **Address :**   **Date of Birth :**

DAILY ACTIVITIES RECORD (WELL)

1. Please make a note of what you were doing and how you felt for the hour before the sample was taken.

2. Please do not take samples for at least one hour after eating food or after brushing your teeth.

3. Remember to rinse your mouth with water before taking samples of saliva.

4. Please take samples of saliva before taking samples of blood.

5. Please put the samples of saliva into your freezer as soon as they have been taken.

TIME OF WAKENING : _____ DATE : _____

TIME	WRITE HERE HOW YOU FEEL AND WHAT YOU WERE DOING
8 a.m.	
11a.m.	
5 p.m.	
8 p.m.	
11p.m.	
8 a.m.	

Appendix 7. Daily Activities Record (unwell)

Name :
Address :

Date of Birth :

DAILY ACTIVITIES RECORD (UNWELL)

For recording how you feel when you are not well.
Take samples if : you feel ill and have to stay in bed.
you have a temperature of 38.5 C or over.

1. Please make a note of what you were doing and how you felt for the hour before the sample was taken.
2. Please do not take samples for at least one hour after eating food or after brushing your teeth.
3. Remember to rinse your mouth with water before taking samples of saliva.
4. Please take samples of saliva before taking samples of blood.
5. Please put the samples of saliva into your freezer as soon as they have been taken.

TIME OF WAKENING : _____ DATE : _____

TIME	WRITE HERE HOW YOU FEEL AND WHAT YOU WERE DOING
8 a.m.	
11a.m.	
5 p.m.	
8 p.m.	
11p.m.	
8 a.m.	

Appendix 8. Protocol for single dose oral 3 day urinary metyrapone test

Protocol for oral 3 day Urinary Metyrapone

Metyrapone Dose:

500mg/m^2. orally, a.m.

Urine collections:

Urine is collected in containers containing no preservative.

1 collection is taken for 24 hours prior to taking metyrapone (8a.m. to 8a.m.)

2 collections 24 hours each after taking metyrapone.

Metyrapone is taken at 8a.m. on day 2.

Assay:

Urinary steroid profiles using Gas-Liquid chromatography.

Appendix 9. Instructions for collecting urine for single dose oral 3 day Metyrapone test

Instructions for collecting Urine Samples for the 3 day Metyrapone Test

1. Collect urine in the container provided (labelled Number 1) over 24 hours.

   Start at a set time in the morning, preferably 8a.m.

   Do not collect the first urine you pass in the morning.

   (When you wake in the morning, pass urine normally in the toilet.

   Start collecting your urine from then on.)

   When you wake up the <u>following</u> morning, collect this first sample of urine as part of the first 24 hour collection.

2. Take the capsule(s) provided as soon as you have finished the first 24-hour urine collection.

3. Collect urine for a further 24 hours in the container labelled Number 2, finishing this collection at 8a.m. the following morning.

4. Collect a final 24 hours of urine in the container labelled Number 3, starting as soon as the previous collection has been completed, and finishing at 8a.m. the following morning.

5. Take all three containers to the Biochemistry lab. with the form provided.

Day 1- urine collection No. 1.	Day 2- urine collection No. 2.	Day 3- urine collection No. 3.
⇩	⇩	⇩
First morning sample - **discard**	Take capsule(s) provided	⇩
⇩	⇩	⇩
Collect all urine passed that day	Collect all urine passed except first morning sample	Collect all urine passed except first morning sample
⇩	⇩	⇩
plus	**plus**	**plus**
⇩	⇩	⇩
First morning sample of next day (Day 2)	First morning sample of next day (Day 3)	First morning sample of next day (Day 4)

Dr. John Schulga.
Research Fellow in Paediatric Endocrinology.

Appendix 10. Insulin Tolerance Test

(Taken from Endocrine guidelines and Investigations, Royal Hospital for Sick Children Glasgow, by Malcolm Donaldson 1996)

This is the gold standard for assessing the ability of the axis to secrete growth hormone. Insulin hypoglycaemia also tests the adrenal axis in a more "physiological" way than the synacthen test. In our hands the ITT is rarely performed in isolation, and is usually combined with a LHRH and TRH test.

(i) The children will be asked to attend at 8.30 - 9.00 am having had nothing to eat or drink (including water) since midnight. Prepubertal children (males and females aged 10 years and over) should be primed with stilboestrol 1 mg twice daily for three days prior to the test. The rationale of this is to "butter up" the hypothalamus, thus enhancing its willingness to stimulate growth hormone release from the pituitary. Please confirm that the child has been fasting, and that he/she has been correctly primed if appropriate. If the child has not been fasted the ITT should not be carried out, and the test rebooked. If the child has not been primed, the test should still go ahead. The weight and height should always be recorded.

(ii) Insert i.v and draw the -30' ("stressed") level. The GH level at this time is important because if stress provokes GH secretion there may follow a refractory period with no GH release in response to insulin. In these cases the initially high GH level confirms adequate GH secretion despite inadequate response to insulin hypoglycaemia.

(iii) Between times -30' and 0' label all bottles clearly and **CAREFULLY READ THE CASE SHEET SO THAT YOU FULLY UNDERSTAND THE CLINICAL PROBLEM.**

(iv) If fasting glucose stix is greater than 2.2 mmol/l proceed with insulin administration using the following doses:

0.15 u/ Kg for children 4 years and over

0.10 u/Kg for children under 4 years.

When there is a high probability of pituitary failure, the doctor arranging the ITT will request a dose of 0.10 u/Kg to be given (check letter).

If initial glucose stix is less than 2.2 mmol/l await formal blood glucose and if this confirms hypoglycaemia perform arginine test, giving i.v. synacthen simultaneously to assess the hypothalamo-pituitary-adrenal axis.

(v) Make up the insulin solution by adding 20 units (0.2 ml) of velosulin to 20 ml saline in a syringe, thus achieving a concentration of 1U/ml.

(vi) At time 0' take further blood samples, then give the insulin (and where appropriate other releasing hormones).

(vii) Check that there is adequate hypoglycaemia in every patient who has an ITT. This means a fall of 50% or more from the fasting blood glucose level, or a minimum level of 2.2 mmol/l.

Do check this by performing glucose stix checks on the sample, and if an adequate fall of blood glucose is not obtained repeat the IV insulin in the dosage of 0.1 U/Kg.

(viii) The procedure for management of hypoglycaemia has already been outlined, but since the achievement of hypoglycaemia is the specific aim of the insulin tolerance test, more detail is given here.

Most patients become drowsy, pale, quiet and sweaty with hypoglycaemia. The nurse or parents should ensure that this child remains conscious during the test, and the child should not be allowed to fall asleep. If glucose stix falls to below 2.2 mmol/l, 10 ml/Kg of Lucozade will alleviate symptoms without affecting GH secretion. If there are profound symptoms of hypoglycaemia including difficulty being roused or trembling, give 50% dextrose (<u>not</u> 10), in the dosage of 0.5g/Kg, i.e. 1 ml/Kg and repeat if necessary to raise glucostix above 4 mmol/l. Do not discontinue sampling if glucose has to be given. Give hydrocortisone phosphate 100 mg if the child remains unwell after glucose administration.

After giving the 50% dextrose and hydrocortisone, re-check the glucose stix, and **do not give more dextrose unless the value is still below 6 mmol/l**. Remember that the hypoglycaemia will not cause death if you act appropriately. It is over enthusiastic correction of hypoglycaemia with hypotonic or hypertonic dextrose that is dangerous, and has contributed to fatalities in children undergoing ITT's.

Protocol for Insulin Tolerance test

Time (mins)	-30	0	15	30	60	90	120
Glucose	+	+	+	+	+	+	+
Growth hormone	+	+	+	+	+	+	+
Cortisol	+	+		+	+	+	+
Thyroxine		+					
TSH		+		+	+		