Glucocorticoid Measurements in Health and Disease – Metabolic Implications and the Potential of 24-h Urine Analyses

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Abstract: For examination of glucocorticoid metabolism and identification of hyper and hypocortisolism, various measurements and diagnostic tools are available. After a brief overview of the physiology of glucocorticoid secretion and glucocorticoid actions, the currently used measurements for blood, saliva, and urine samples and the corresponding physiological and metabolic implications are critically reviewed. A special emphasis is placed on the potential of 24-h urine analyses to assess not only glucocorticoid secretion, but also functional glucocorticoid activity.

Key Words: Glucocorticoid measurements, cortisol, cortisone, urinary free steroids, functional glucocorticoid activity, 11beta-hydroxysteroid dehydrogenase, 5alpha-reductase, 24-h urine collection, children.

INTRODUCTION

After a brief description of (I) the physiology of glucocorticoid (GC) secretion, (II) normal GC actions along with symptoms of GC deficiency and excess, (III) and their metabolic inter-conversions, the currently used measurements are reviewed with regard to the kind of sample types (saliva, blood, urine) under study and the corresponding physiological and metabolic implications. In this mini review emphasis will be placed on specific aspects and limitations of GC measurements in blood samples and the potential of GC metabolite analyses in urine samples. Using 24-h urine collections renders possible to examine in the same sample i) adrenal GC secretion rate, ii) potential functional GC activity (i.e. an indicator for the GC fraction that potentially enters tissues and cells to exert cortisol-dependent actions), and global activities of steroid metabolising enzymes. However, for GC measurements in urine samples various methodological pitfalls lurk.

I. GLUCOCORTICOID SECRETION

Glucocorticoids (GCs) comprise a group of steroid hormones with a C21 steroid structure of which the C-atoms at the positions 11, 17, 20, and 21 are oxygenated. These hormones are essential for life and are secreted by the adrenal gland under the control of the hypothalamus and the pituitary gland. In humans, cortisol is the primary glucocorticoid. It is synthesized and released into blood from the zona fasciculata of the adrenal gland. Apart from cortisol, several of its metabolites like 6beta-hydroxycortisol, 20alpha- and 20beta-dihydroxycortisol, and cortisone are also directly secreted by the gland, but their concentrations in the adrenal vein are much lower than that of cortisol [1-3]. A very small fraction of the direct secretion products are even released from the adrenals sulfated at position C20 of the glucocorticoid molecule [1]. Studies on patients – mostly with isolated mineralocorticoid secretion disorders – have demonstrated a molar relationship of ≈ 1:10 for cortisone to cortisol in the steroid enriched adrenal vein blood, indicating that cortisone is a quantitatively, not to be ignored, direct adrenal secretion product. In line with this, are observations in the older literature that showed a cortisone concentration gradient from adrenal vein to peripheral vein of ≈ 3:1 (for literature see Walker *et al.* 1992 [4]).

Peripheral blood cortisol, but not necessarily peripheral blood cortisone levels [4] increase after an activation of the hypothalamic-pituitary-adrenal (HPA) axis. The reason for this seemingly unresponsiveness of circulating cortisone will be discussed below. HPA axis activation is brought about by amplification of specific neuronal signals to the hypothalamus and systemically or locally elevated cytokines caused by stressful conditions as, for example, hypoglycemia, hypoxemia, hypotension, pain or injuries. The hypothalamus processes these signals and responds with an increased release of corticotropin-releasing hormone (CRH). In the anterior pituitary, CRH stimulates secretion of adrenocorticotropic hormone (ACTH), which in turn stimulates GC secretion. If circulating cortisol increases above the usual level, it feeds back to the HPA axis and slows down GC secretion.

Despite this closely regulated mechanism, that controls cortisol secretion within relatively narrow limits, total daily cortisol secretion rates vary considerably between individuals which can be inferred from the large inter-individual variabilities observed in studies on cortisol production rates. A considerable degree of heritability for cortisol secretion appears to be responsible for this inter-individual variation, as has been suggested from genetic studies primarily performed on cortisol plasma levels [5, 6]. However, heritability studies on direct adrenal steroid hormone output are lacking. Such direct measurements of GCs in adrenal vein plasma are normally limited to patients catheterized for specific diagnoses, but are ethically not justifiable in healthy subjects, espe-

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cially children. Therefore, measurements of cortisol in plasma and serum samples – with and without particular endocrine stimulation or suppression test – are frequently used as an index for the cortisol secretion status. However, this implies a number of limitations and pitfalls.

II. MAJOR PHYSIOLOGICAL GLUCOCORTICOID ACTIONS AND SYMPTOMS OF DEFICIENCY AND EXCESS

Physiology

GCs play a key role in regulating relevant steps of metabolism of the major energy-providing nutrients protein, glucose, and fatty acids. GCs increase blood glucose levels and facilitate the delivery of glucose to cells and tissues not only during stress, but also with increasing time after energy and nutrient ingestion. The rise in glucose release into circulation results from GC-induced increase in the rate of hepatic gluconeogenesis and inhibition of adipose tissue glucose uptake. GCs also stimulate free fatty acid release from adipose tissue and amino acid release from body proteins. GCs are involved in blood pressure regulation, salt and water metabolism and are required for normal cardiovascular reactivity to numerous stimuli. They essentially enhance the synthesis and secretion of catecholamines in the medulla of the adrenal gland. GCs possess anti-inflammatory and immunosuppressive effects by affecting most cells that participate in immune and inflammatory reactions, including lymphocytes, natural killer cells, neutrophils, mast cells, and basophils [7].

Deficiency

In essence, GCs are particularly important at times of stress, when they provide a longer-term signal to damp many of the acute responses to illness and "reset" metabolism in favor of providing substrates for oxidative metabolism [8]. The biological essentiality of GCs is best exemplified in the clinical syndromes of deficiency (adrenal insufficiency due to Addison's disease or hypopituitarism). The major symptom of chronic insufficiency is fatigue, accompanied by lack of stamina, loss of energy, reduced muscle strength, and increased irritability [9]. Weight loss, nausea, anorexia, postural hypotension, hypoglycemia, and in infants the failure to thrive occur in addition as mostly unspecific symptoms. In case of strong stress, severe illnesses, or multiple traumas, a hypodynamic shock can threaten life in undiagnosed Addison patients or patients with partial adrenal insufficiency [9].

Excess

Endogenous cortisol excess is mainly caused by an ACTH-producing pituitary adenoma (Cushing's disease), by an adrenal tumor (Cushing's syndrome), or by an ectopic ACTH-secreting tumor [10]. The hypercortisolism is characterized by central obesity, abdominal purple or red striae, hypertension, edema, and glucose intolerance. In the classical phenotype, fatness predominates as characteristic regional fat pads [11] in the face ("moon face"), at the trunk, and on the back of the neck (buffalo hump). Osteoporosis or osteopenia [12] and growth inhibition [13] as well as brain atrophy, memory impairment, and depression [14] are additional symptoms frequently seen in Cushing's syndrome. Apart from GC replacement therapy in adrenal insufficient patients, GCs are also heavily used in treating non-endocrine disease, often associated with adverse side effects. Common co-morbidities are growth suppression (in children), excess weight gain, and osteopenia [15].

III. CORTISOL-CORTISONE SHUTTLE

The enzyme 11b-hydroxysteroid dehydrogenase (11b-HSD) catalyses the reversible interconversion of cortisol to its hormonally inactive 11-oxo metabolite cortisone [16-19]. Two isoforms of the enzyme exist 11b-HSD1 and 11b-HSD2 [20, 21]. 11b-HSD1 is expressed in many cell types and organs, but quantitatively the most important organs are probably the liver and the adipose tissue [22]. Its predominant *in vivo* reaction direction is the reduction of cortisone to cortisol, i.e., the reactivation of the 11-keto corticosteroid to the active glucocorticoid. With this reaction, a locally high cortisol production is possible from the substantial circulating levels of cortisone [23]. *In vivo* studies in humans and knockout models in mice strongly suggest that this enzyme effectively amplifies glucocorticoid action in liver, adipose tissue, and brain. Inhibition of 11b-HSD1 by glycyrrhetinic acid, a relatively unselective liquorice-based inhibitor, is associated with features of reduced glucocorticoid action in the liver and increased hepatic insulin sensitivity [23]. Expression of the enzyme in characteristic brain regions such as hippocampus, hypothalamus, and pituitary suggests that it participates in negative feedback regulation of the HPA axis by endogenous glucocorticoids. Correspondingly, 11b-HSD1 null mice show adrenocortical hypertrophy and increased responses of the adrenal gland to ACTH since the intrahypothalamic generation of cortisol and its subsequent binding to the receptors (glucocorticoid and mineralocorticoid receptors) is impaired [23].

Basically, large quantities of the cortisone, that has been converted from cortisol by the second 11b-HSD isoenzyme, the 11b-HSD2, are reconverted by 11b-HSD1 to cortisol. As mentioned, liver and adipose tissue play the quantitatively most important role in this process of cortisol regeneration which has been termed cortisol-cortisone shuttle (Fig. **1**) [19]. The body's major supplier of substrate for this shuttle is the kidney with high 11b-HSD2 activities in the collecting ducts.

Apart from the kidney, the high affinity enzyme 11b-HSD2 is also considerably expressed in the human colon and salivary gland. In these organs, especially in the kidney, 11b-HSD2 catalyses dehydrogenation of cortisol to cortisone to protect the mineralocorticoid receptors from inappropriate activation by cortisol. *In vitro*, the mineralocorticoid receptor has equal affinity for aldosterone and cortisol and inactivation of the latter allows appropriate binding of aldosterone to ensure the selective reabsorption of sodium in the distal nephron as well as appropriate renal excretion of potassium [17, 23]. The importance of this enzyme is illustrated by the clinical consequences of its deficiency. Mutations in the gene encoding 11b-HSD2 account for an inherited form of hypertension, the syndrome of Apparent Mineralocorticoid Excess where cortisol induces hypertension and hypokalaemia [21, 24].

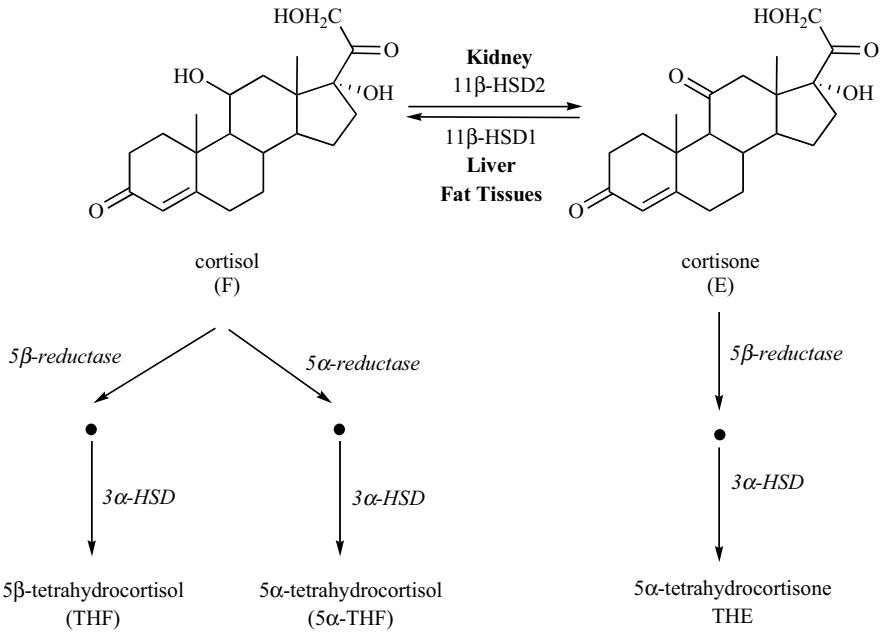

Fig. (1). Metabolism of cortisol and cortisone and formation of the three major catabolic end products.

IV. GLUCOCORTICOID MEASUREMENTS IN BLOOD SAMPLES

Several measurements and methods to evaluate adrenocortical function are in use. The combined measurement of early morning serum cortisol and plasma ACTH can separate patients with primary adrenal insufficiency from healthy individuals and from those with secondary disease [9]. Other approaches encompass stimulation or suppression tests, with administrations of ACTH (low-dose or standard-dose corticotropin test), insulin (insulin tolerance test) or metyrapone (an inhibitior of 11beta-hydroxylation of adrenal cortisol synthesis). All of these tests, including the exogenous GC administration for diagnosis of Cushing's syndrome (dexamethasone suppression test), have particular implications and limitations [9, 10, 25], of which evaluation would be beyond the scope of this paper.

As a simplified clinical practice to assess adrenal function in critically ill patients, the examination of random serum total cortisol levels is advocated [7, 26]. This approach has been shown to provide reasonable prognostic information in the specific condition of severe stress due to hypoxemia, sepsis, or multiple trauma. During such extreme stress situations, random serum total cortisol levels are even superior to most endocrine function tests, as has been explained in detail by Marik and Zaloga [7] and Arafah [26].

The usual way to obtain "normal" baseline glucocorticoid serum levels in the healthy subjects or less ill patients is to collect fasting blood samples in the morning or to withdraw blood at certain intervals over periods of up to 24 hours. The latter is required for analysis of prolonged hormone profiles and circadian hormone variations. In observational and epidemiological studies, measurements of baseline cortisol serum levels are frequently used as an index of adrenocortical function [6, 27, 28] because dynamic exploration of adrenal function with endocrine stimulation tests is not possible or too elaborate. This approach presumes that basal cortisol concentrations and stimulated cortisol concentrations (after endocrine manipulation) are largely associated. Although this is often the case [26, 29, 30], the simple conclusion that high serum concentrations of cortisol reflect high adrenocortical activities and low serum concentrations reflect low secretory activities does not apply in a number of physiological and pathophysiological situations, even not after the endogenous cortisol secretion is stimulated by ACTH or by an insulin-induced hypoglycaemia.

Cortisol plasma levels can even be reduced in situations with elevated adrenocortical activity. Although frequently overlooked, a typical condition showing this metabolic constellation is conventional obesity (BMI > 30 kg/m^2) in adults. Most studies that reported data on adrenocortical activity in people with elevated body fatness found increased cortisol productions or secretions [31-34], only a few found unchanged values (e.g., [35]), and – to our knowledge – not a single publication reported a reduced cortisol production in otherwise healthy obese. However, circulating cortisol is frequently reduced with obesity, in morning fasting blood samples [36-39] as well as after a noontime meal [37] in females and in basal blood samples collected at various times over the day [29] as well as after iv insulin administration in males [29]. The authors of the latter publication interpreted their results as compelling evidence for a hypocortisolemia and decreased cortisol secretion in obesity, although they did not measure markers for cortisol secretion, e.g., urinary excretion rates of major GC metabolites (see below). In addition, the usual impact of the obesity-related hyperinsulinism on the major binding protein for cortisol in human plasma, the corticosteroid binding globulin (CBG), was not consid-

ered. Thus, conventional cortisol measurements in blood samples (plasma or serum) can lead to considerable misinterpretations. The primary cause for such misinterpretations (A), the endocrine and metabolic background factors (B), and possible blood-based measurement alternatives (C, D) are discussed in the following sections.

A. Varying CBG as Confounder of Basal and ACTH-Stimulated Blood Total Cortisol Concentration

Cortisol circulates in plasma in three states: less than 5 % being unbound, i.e., free to rapidly cross cell membranes and interact with the steroid receptors; about 90 % bound to CBG; and up to 10 % bound to albumin [26, 40]. The major transport protein, CBG binds glucocorticoid hormones with high affinity thus regulating cortisol's bioavailability by restricting exit from capillaries. It is generally accepted that the CBG-bound cortisol has a restricted access to target cells [41].

Due to the overall very high protein binding of circulating cortisol, it is clear that total serum cortisol levels are affected by variation in plasma proteins, especially CBG. The importance of a fall in serum CBG on total serum cortisol was recently recognized in critically ill patients [42]. Basically, Arafah [26] reviewed the corresponding results as follows: It could be shown that the usual ACTH-stimulated increase in serum total cortisol concentration is not discernible in patients with reduced albumin and CBG levels despite proved normal adrenal function. These patients showed appropriate increases in the free, immediate bioavailable cortisol fraction. Correspondingly, such critical ill patients would have been falsely classified as having adrenal insufficiency, if only total cortisol levels were measured. Thus, evidence is now overwhelming that serum total cortisol concentrations and their increments strongly depend on the circulating CBG concentration [26, 42]. CBG is relatively rapidly saturated with cortisol. At physiological CBG concentrations, this protein binds up to 25 μg/dl (690 nmol/L) of the circulating cortisol. If CBG is reduced, a sudden ACTH-induced rise in cortisol secretion can markedly exceed the protein binding capacity, thus leading to an overflow into the unbound (free) cortisol fraction. Since the latter, having a short half-life, is rapidly metabolised, serum total cortisol level shows only small (seemingly inadequate) increases, or relative reductions compared to normal or high CBG subjects.

Increasing evidence strongly suggests that this confounding relationship holds true also in normal subjects. Recently, Dhillo *et al.* [43] found that total serum cortisol, whether quantified in the basal state or after ACTH stimulation (250 μg Synacthen test), correlated significantly with healthy female and male volunteers' CBG levels. Accordingly, in an earlier study Bright and Darmaun [44] showed that total blood cortisol measurement was likely to underestimate adrenal function in subjects with lower CBG. The authors infused known amounts of [9,12,12-2H3]cortisol to subjects with a suppressed endogenous cortisol secretion in order to simulate certain cortisol productions rates. However, a reasonable prediction of plasma cortisol responses was only achieved after individual CBG concentrations were accounted for.

B. Estrogens, Growth Hormone, Insulin, Obesity, and Inheritance Affect CBG

Numerous factors affect serum CBG and thus serum cortisol levels. Among them are illnesses and also various drugs and medications [26]. For example, oral contraceptives and estrogens increase hepatic CBG production [26, 45]. Consequently, total cortisol plasma levels are clearly elevated in women taking oral contraceptives with a relevant oestrogen content compared to men without medication (Fig. **2**). However, the oestrogen stimulus on CBG does not result per se in an altered stress activity or urinary cortisol excretion [46]. Also pregnant women show elevated total plasma cortisol levels [26, 47].

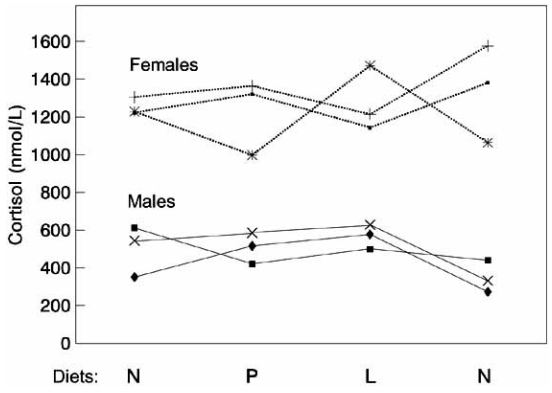

Fig. (2). Total plasma cortisol in healthy males and females on a normal mixed (N), a protein-rich (P), and a lacto-vegetarian (L) diet [46]. Females were users of estrogenic pills.

Additional physiological factors influence CBG levels. Both growth hormone [48] [49] and insulin [50, 51] decrease CBG, implying that the unbound (bioavailable) fraction of cortisol may increase. Especially if total circulating cortisol remains constant, a reduction in CBG means an increase in free cortisol, which – as a trend – has actually been observed after growth hormone replacement [48] and in normal, mostly non-obese subjects (mean BMI ≈ 25 kg/m^2) with decreasing insulin sensitivity, i.e. increasing insulin secretion [50, 51].

The physiological increase in insulin secretion occurring along with the decreasing insulin sensitivity in otherwise healthy people who grow fatter can thus explain why finally in the obese subjects the lowest CBG levels develop [52]. This low circulating CBG concentration will then, in parallel, result in reduced total plasma cortisol concentrations [26, 43] (see section A). Thus, the seemingly paradox, simultaneous occurrence of reduced total plasma cortisol and elevated adrenal cortisol secretion appears to be a normal phenomenon in conventional obesity in adults which should not be misinterpreted as a hypocortisolism. In line with the reduced CBG levels as major cause for low total plasma cortisol are additional findings of Jessop *et al.* [29]. The authors observed that even after cortisol infusion and insulin injection, total plasma cortisol remained reduced in the obese adults

compared to normal weight controls at certain periods of the day.

In contrast to conventionally obese adults, obese children with insulin resistance have been reported to show slightly increased total blood cortisol concentrations [53]. During growth the metabolic situation is further complicated by additional influencing factors, e.g., by the so called physiological insulin resistance occurring around puberty. Even in the case of reduced CBG levels, total serum cortisol may increase (without a change in cortisol blood production rate) if its binding to other binding proteins, especially serum albumin, strongly increases. Several studies have shown enhanced binding affinities of steroid hormones (including corticosteroids) to albumin by binding of increasing amounts of free fatty acids (FFAs) to albumin (for literature, see reference: [54]). FFA concentration increases especially during short-term fasting (with consequences for morning fasting blood samples) and this increase may be particularly high in children with a physiological insulin resistance and an additional obesity-related insulin resistance. Apart from this potential modulating effect of an altered serum protein binding capacity through varying FFAs on total serum cortisol, the influence of variation of CBG itself appears to be of major importance. However, highly sophisticated research studies on changes in total serum steroid hormone concentrations should not only consider the specific binding proteins like CBG, but also albumin and FFAs as potential confounders.

The relevance of CBG as a confounder of total plasma cortisol measurements can also be deduced from the fact that CBG levels in individuals are determined, at least in part, by inheritance [41, 55] and accordingly, vary significantly between individuals [43]. This can explain why those with the lowest CBG in circulation frequently also show the lowest responses to ACTH (e.g., in the short Synacthen test) which implies an increased risk of being falsely diagnosed to have an inadequate HPA reserve [43].

C. Serum Cortisone

For cortisone, the 11-keto metabolite of cortisol (Fig. **1**), binding to circulating CBG is clearly less important than for cortisol itself [8, 40]. The moderate CBG binding of cortisone can explain, in part, the markedly lower total circulating levels of around 50 - 100 nM compared to about 400 nM on average for cortisol. The fraction of cortisone that circulates free, i.e., unbound to any plasma protein is relatively high, i.e., around 10 nM which compares with free plasma cortisol of ≈ 10 nM in the evening during a normal circadian rhythm [8]. This reduced plasma protein binding of cortisone assures an ample supply of the substrate cortisone for re-activation to cortisol by 11b-HSD1 [8]. In line with such an ample supply of "pre-cortisol" to liver and peripheral tissues may be the observation of Walker *et al.* [4] that no strong circadian rhythm exists for total plasma cortisone. Hepatic 11b-HSD1 appears to effectively convert the unbound cortisone, which is flooding from the kidney to the liver, to cortisol [4]. Accordingly, administration of cortisone by mouth, which is delivered to the liver by the portal circulation, results in high circulating total cortisol, but not in high circulating total cortisone concentrations [8]. The aforementioned confirms thus for cortisone, what has already been elucidated for cortisol, namely that measurement of total circulating glucocorticoids does, in many situations, not provide an adequate reflection of the current bioactivity and/or production level of the respective corticosteroid. The above observations also call into question whether the ratio of total plasma cortisol to total plasma cortisone can really be used to appropriately assess physiological variation of 11b-HSD 1 or 2. Furthermore, an additional argument against a one-time blood measurement of circulating glucocorticoids in healthy subjects may be seen in the high day to day variability that has been reported to vary around 26 % for total plasma cortisol [56]. On the other hand, with respect to a practicable clinical assessment of pathophysiologically relevant alterations in GC metabolism or secretion in different patient groups, combined measurements of serum GC metabolites (e.g., cortisone and cortisol) may be useful [57, 58]. Also the additional quantification of circulating metabolites, hitherto not used for routine analyses, may be considered.

D. Serum Free Cortisol Measurement and Functional Cortisol Status

Measurement of serum free cortisol concentration appear to be most appropriate approach for assessing adrenal function in critically ill patients, who often have reduced CBG and albumin levels and consequently reduced total serum cortisol concentrations, even after ACTH stimulation [26, 59]. The decreased total binding of cortisol to CBG in critically ill patients results in larger albumin-bound and free proportions [26], of which overall cortisol concentrations are reduced due to simultaneously lowered albumin and the – per se – high metabolic clearance of the unbound fraction. However, despite this lowered total cortisol, the free, bioavailable fraction can be appropriate for the stress situation. On the other hand, in patients with actually impaired adrenal function, the gland's cortisol secretion is no longer sufficient to adequately saturate the usual proportion of circulating CBG, which results in inappropriately low serum free cortisol levels. It is therefore plausible that the appropriateness of HPA axis activation can be reliably assessed by serum free cortisol measurements. In line with this, are the findings of Dhillo et al. [43] suggesting that healthy subjects with individually (probably genetically) lower CBG levels and correspondingly subnormal serum total cortisol responses after ACTH stimulation, have normal increases in an index of serum free cortisol. However, some questions remain to be clarified before serum free cortisol can be recommended as the preferable parameter to invasively assess the functional cortisol status not only in pathophysiological but also in physiological conditions: is a single blood sample enough (secretory bursts, circadian rhythm), or are multiple blood collections required, and at what time (morning, midnight ?) or over what period of time should be examined? In an older study, Friedman and Yanovski [60] observed a lower specificity of 8:00 a.m. plasma free cortisol, compared with 8:00 a.m. plasma total cortisol and 24-h UFF, in the diagnosis of Cushing syndrome. Since current assays for determining serum or plasma free cortisol concentrations are difficult, time consuming, and labour intensive [26], the availability of adequate routine assays in the near future will help to get the answers.

As has to be pointed out, the above HPA function assessment by serum free cortisol measurement does not mean that adrenocortical activity is "automatically" assessed too, since a normal or even reduced adrenal cortisol secretion (adrenocortical activity) can be present despite increased functional cortisol activity or increased bioavailable cortisol. This is the case, for example if peripheral cortisol clearance is diminished.

V. CORTISOL IN SALIVA SAMPLES

Several groups have examined the potential of salivary cortisol concentrations as a surrogate marker for serum free cortisol levels. In general, high positive correlations of salivary cortisol with unbound plasma cortisol have been reported [26]. This is obviously due to an existing equilibrium of the former with the later [26]. Salivary cortisol measurements are frequently used to evaluate states of glucocorticoid excess, not only in patients with a potential Cushing's syndrome [12], but also in psychological tests and physiological acute studies. For example, in 1999 Gibson *et al.* [61] studied the cortisol response to different dietary intakes using the stress-free technique of saliva collection before and after midday meals. The authors found increasing cortisol after a high protein meal, but not after a low protein meal and observed that the extent of this increase correlated with poor psychological well-being. Salivary cortisol proved to reflect free serum cortisol levels independent from plasma binding proteins and it also responds sensitively to low dose ACTH stimulation (1 μg). It can be concluded that salivary cortisol measurements are a reliable and practical measure of the serum free cortisol concentration since they are simple to obtain and easy to quantify in most laboratories. However, one drawback can be that a single saliva sample – similar to a single serum free cortisol measurement – only reflects the acute situation in which the sample has been collected, so that further conclusions on overall functional cortisol status may be misleading. In this regard, serious problems with subjects' compliance have been observed after home salivary collections were stipulated at predetermined time points [62].

Apart from cortisol itself, additional metabolites have been quantified in saliva, of which 6b-hydroxycortisol (6b-OHF) appears to be a promising alternative [63], since it is a polar molecule that needs neither conjugation with glucuronic acid nor with sulphuric acid [64]. In addition, 6b-OHF varies in parallel with cortisol in plasma and saliva samples [63] and its urinary excretion may better reflect stress-full conditions than urinary free cortisol [64], provided thyroid function is normal [65].

VI. GLUCOCORTICOID ANALYSES IN URINE SAMPLES

A. Assessment of Daily Glucocorticoid Secretion, i.e., Adrenocortical Activity

In the urine of adults, between 72 % and 88 % of the radioactivity injected as a single tracer dose of cortisol, either labeled with ^{14}C or with ^{3}H, is recovered within 24 hours [66]. After 48 hours recovery is 90 % on average [66]. The 7 major urinary cortisol metabolites 5α-tetrahydrocortisol (5a-THF), 5β-tetrahydrocortisol (THF), 5β-tetrahydrocortisone (THE), α-cortol, β-cortol, α-cortolone, and β-cortolone, frequently termed total urinary cortisol metabolites (C21) encompass almost 80 % of the cortisol secreted by the adrenal gland [67]. The sum of 5a-THF, THF, and THE (GC3) encompass 50 % of total GC secretion [67]. For this reason, a 24-h urinary collection for measurement of cortisol metabolites provides a time integrated, stress-free, *in-vivo* result of the amounts of cortisol and cortisone originally secreted by the adrenal gland. Accordingly, measurement of urinary 24-h glucocorticoid metabolite excretion is an established method, used by numerous endocrine research groups [24, 65, 68-72] to examine glucocorticoid metabolism and/or adrenocortical activity in healthy and ill children and adults. For example, a sex dimorphism with higher GC secretion rates in males than females has been demonstrated with this method [34, 73, 74] as well as a frequently increased adrenocortical activity in obese subjects [31-34].

B. Overall Potential of Urinary Glucocorticoid Measurements

The advantage over single total serum cortisol measurements (and serum measurements repeated over only short periods of few hours) is that the urinary results are neither affected by short-term fluctuations and sudden secretion bursts of cortisol nor by varying plasma protein binding capacities. They integrate the produced metabolites over a 24-h period. The non-invasive character of urine measurements is of considerable ethical importance for children and especially for healthy children in whom repeated hormone analyses are planned. In addition, 24-h urine collections allow a particular, complex examination of the overall glucocorticoid status which is not possible with usual glucocorticoid measurements in blood or saliva samples. The complex examination includes a simultaneous differentiation between i) the glucocorticoid secretion (adrenocortical activity) *via* C21 or GC3 analyses, ii) the level of potentially bio-active glucocorticoids, i.e., the functional cortisol status (or stress activity) *via* urinary free cortisol and cortisone measurements (see sections F and G), and iii) the underlying overall steroid hormone enzyme activities, i.e., glucocorticoid metabolism *via* examination of specific metabolite relationships.

As already mentioned in chapter IV (section D), an elevated stress activity, i.e., an elevated bioavailable glucocorticoid level can be present without a parallel increase in adrenal cortisol secretion, if metabolic glucocorticoid clearance (i.e., cortisol and/or cortisone degradation) is decreased. The stress activity can be identified with serum free cortisol or saliva cortisol measurements, but these measurements do not allow to identify the respective level of adrenal glucocorticoid secretion or the potentially underlying metabolic enzyme activities. Examples for conditions with varying adrenocortical activities and the corresponding specific urine metabolite examinations are given in section J.

Day-to-Day Variation

For measurements of total plasma or total serum cortisol a relatively high day to day variation ranging from 19.5 % [75] to 26 % [56] (within-subject and analytical variation combined) has been reported for healthy adults. When determining the day-to-day variability in urinary excretion rates of GC3 in serial measurements of 24-h urine samples collected on 3 consecutive days in 10 healthy children aged 9 -

11 years (5 girls), we found a markedly lower mean variation of 12.4 % (Fig. **3**). This relatively low day-to-day variation in respect of glucocorticoids, although higher in comparison to daily renal creatinine output, displays a potential additional advantage of 24-h urine corticosteroid analyses, apart from its non-invasive character and the possibility to differentiate between secretion, potentially bio-active fraction and metabolism of GCs.

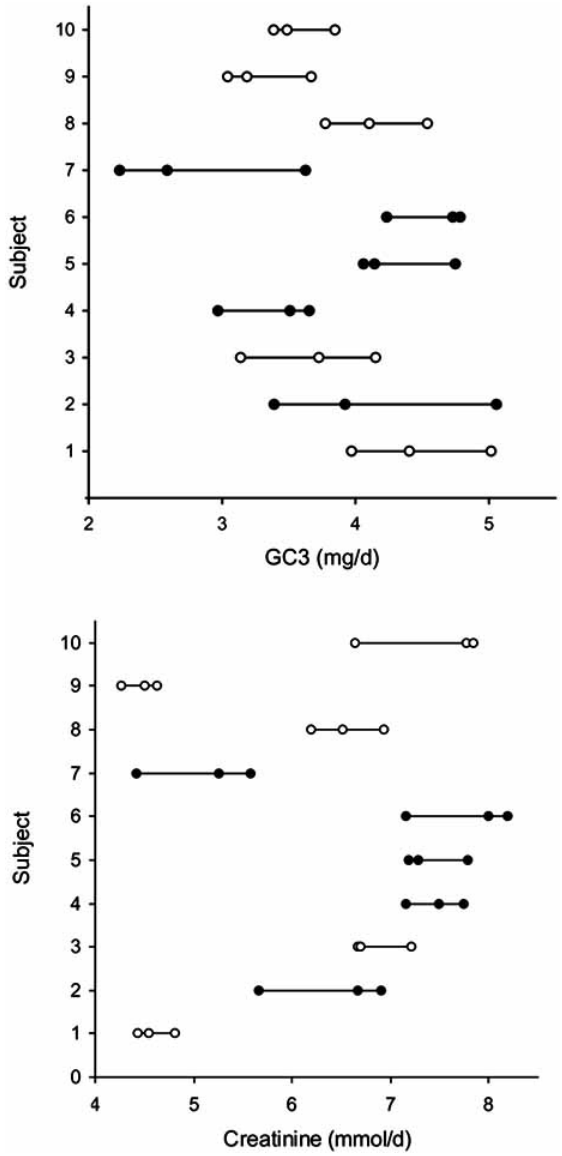

Fig. (3). Day-to-day variability in urinary excretion rates of GC3 in serial measurements of 24-h urine samples collected on 3 consecutive days in 10 healthy children aged 9 - 11 years (5 girls) in comparison with urinary creatinine excretion.

C. Limitations of 24-h Urine Collections

As with other measurements and samplings – also urine measurements and 24-h collections have limitations. They are some what cumbersome, time-consuming, and the procedure has to be explained in detail to patients and probands. Collection errors and insufficient compliance of 24-h urine collections can occur. Therefore, it is essential to analyse urinary creatinine excretions in all samples and to perform checks on collection compliance [10], e.g., by using body weight-corrected 24-h urinary creatinine excretion reference values [76]. Furthermore, a clear written guidance for 24-h urine collection should be provided along with a form where all micturition times and particularities during the 24-h collection should be filled in. Ideally, but often not possible, a short interview on compliance and correctness of the filled out form should complete the sampling.

D. Use of Analyte Creatinine Ratios

For spot urine samples, it is a matter of routine that numerous analytes are normalized to creatinine. By multiplying such analyte/creatinine ratios with sex- and age-specific body weight-related creatinine reference values [76] it is possible to reasonably estimate actual 24-h excretion rates of many analytes for groups of subjects (not for an individual) simply from spot samples. This epidemiological approach has been successfully applied also to hormones, for example dehydroepiandrosterone sulphate [76], but – as expected – has proven inapplicable for a useful estimation of the daily output of urinary free cortisol [76]. Obviously, the impact of the circadian rhythm and the spontaneous increments in cortisol secretion do not allow a reasonable estimation of 24-h cortisol excretion from spontaneous urine samples. Kong *et al.* [77] who determined the morning cortisol/creatinine ratio minus the midnight cortisol/creatinine ratio to examine the midnight to morning urinary cortisol increment, found this more complex approach, covering a defined time period, to be an accurate non-invasive tool for the assessment of the HPA function.

Apart from the particular use of creatinine for defined time periods and apart from the epidemiological use of creatinine to standardize numerous urinary analytes in spot samples, some authors prefer to calculate the analyte to creatinine ratios also for 24-h urine collections, instead of providing the absolute daily excretion rate for the respective analyte. However, this ratio can have markedly higher variations than that for either variable considered alone, especially in children [78, 79]. The reason for this can be seen in the fact that – physiologically (i.e., in healthy subjects) – the renally excreted absolute amounts of many analytes (e.g., organic acids, net acid excretion, urinary free cortisol, iodine, sodium, magnesium) are closely related to body size, energy metabolism and energy intake, of which variation is best reflected by variation in body surface area (BSA) [79-83]. However, muscle mass and thus urinary 24-h creatinine excretion show markedly steeper increases during growth than energy intake and BSA [76, 84]. Accordingly, the analyte creatinine ratios of most urinary variables, including that of urinary free cortisol [76, 82], decline during growth. In line with this, many researchers [10, 78, 82], including the presenting authors (see section F) did not find the ratio of cortisol to creatinine to be a useful index for cortisol status assessment. Creatinine correction can even mask the normal adrenarchal increase in urinary 17-ketosteroid androgen sulphate excretion [85].

If however, different urinary analytes, measured in the same urine samples, shall be regressed on each other then the analyte creatinine ratios should be calculated even for 24-h

urine samples. This approach becomes necessary specifically for statistical analyses to avoid correlated measurement errors which can occur since not all subjects may collect exactly for 24 hours. Those cases with a shorter (or longer) collection period will have per se correspondingly lower (or higher) total excretion rates of the variables to be compared. The use of the analyte creatinine ratio allows to exclude erroneous correlation results between variables from the same samples (in simple, multiple and multivariate regression models) that could originate plainly from collection errors. Multiplication of the 24-h analyte creatinine ratios with sex- and age-specific creatinine reference values [76], for both dependent and independent variables before regression analysis, even allows to use a less abstract quite accurate estimate of 24-h excretion rates instead. Fig. **4** shows as an example, the excellent correlation between the measured actual daily excretion rate of urinary free cortisol (UFF) and its estimated daily excretion as yielded by multiplication of each individually determined 24-h UFF creatinine ratio by individual body weight and published sex- and age-specific body weight-related creatinine reference values [76].

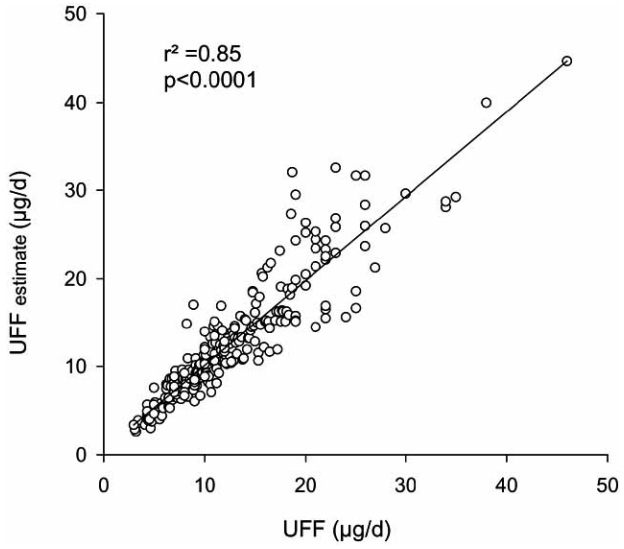

Fig. (4). Correlation between actual 24-h urinary free cortisol (UFF) excretion and an estimate of 24-h UFF excretion determined using the UFF/creatinine ratio.

Fig. **5** shows the increase in absolute daily excretion rates of the sum of the 3 major urinary glucocorticoid metabolites 5a-THF, THF, and THE (GC3) in healthy children aged 4 - 14 years. If these values are related to daily creatinine excretion, a clearly decreased ratio is discernible for the ages 8-9 and 12-14 years compared to age 4-5 years (Fig. **5**). This lower level in older children – being in line with many other analyte creatinine ratios [76, 79, 86] – must not be misinterpreted to be indicative of a reduced metabolic need for cortisol in adolescents compared to 5 years olds. 5 year old children on GC replacement therapy do not have higher daily GC needs than adolescents [87-89]. After normalisation of daily urinary GC3 excretion to BSA (GC3/BSA) and after considering that daily urinary GC3 encompasses 50 % of total GC secretion, a physiologically BSA-related need of about 10 mg of cortisol (10 mg/day/m^2) can be deduced for

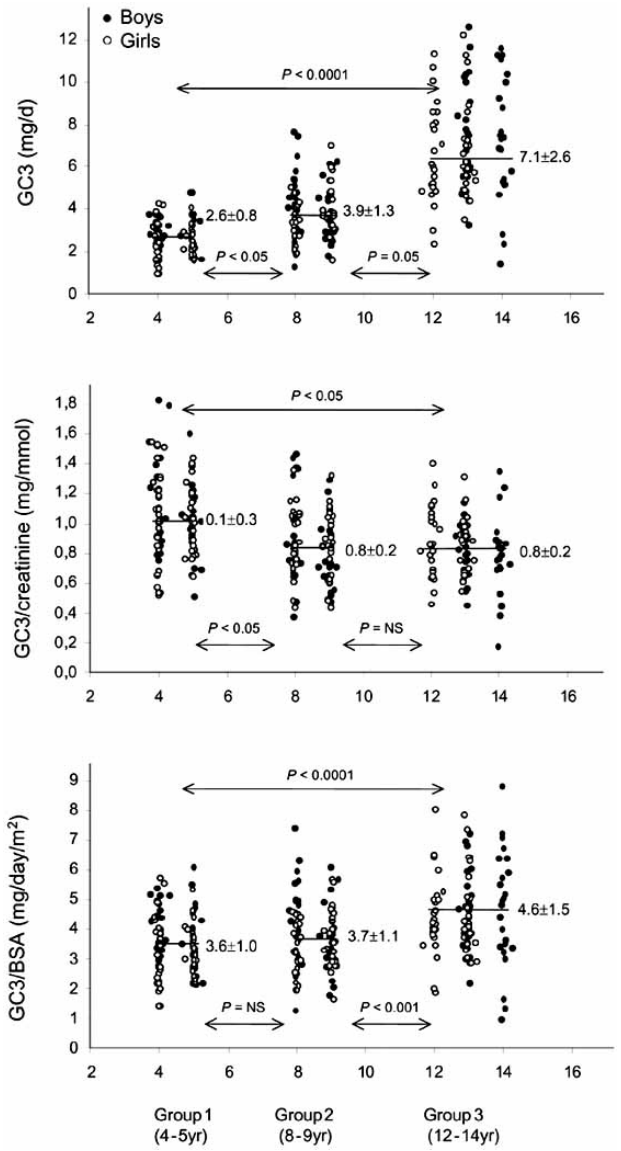

Fig. (5). Renal 24-h excretion rates of the sum of the three major urinary glucocorticoid metabolites (GC3) reflecting approximately 50 % of the daily adrenal glucocorticoid secretion. Absolute, creatinine-corrected, and body surface area-adjusted data from 300 healthy children [95].

the 12-14 years old adolescents from the data of Fig. **5** (4.6 mg/day/m^2 x 2). The actually recommended treatment dose for children with cortisol synthesis defects is 10 - 20 mg/m2 per 24 hours [87-89] and recent findings indicate that an average of 17.6 mg/day/m2 may already be too high, since negative side effects on height development are discernible [90].

E. Anthropometry-Based Urinary Excretion Rates

Correcting urinary cortisol (or other variables, that are also physiologically related to energy intake and body size) by body mass index (BMI) would not yield useful ratios too, since BMI is primarily an index of fatness and as such,

higher BMI levels rather integrate a preceding excess of energy intake than reflect actual energy needs. The inappropriateness of this ratio can also be deduced from the fact that average BMI values of children show a nadir around 5-6 years (adiposity rebound) which would create cortisol/BMI variations with age which are neither associated with growth processes and energy needs, nor with cortisol secretion. A frequently used anthropometric correction parameter for urinary 24-h excretion variables is BSA, as already mentioned in the last section. Correction of metabolic parameters with BSA accounts for most of the variability due to variation in body size-related energy intake and allows age-independent comparisons of daily excretion rates between young children, adolescents, and adults for many urinary analytes, for example, organic acids [80], net acid [81], magnesium, sodium [83], and iodine [79], but not for GC3, i.e. cortisol secretion (Fig. **5**). Daily cortisol secretion and thus the metabolic need for cortisol (related to BSA) appear to be significantly higher during puberty than during childhood. These physiological phenomenon has been recently confirmed in healthy children with the gas chromatography-mass spectrometry reference method for measuring urinary steroid metabolites [91].

F. Urinary Free Cortisol and Non-Invasive Assessment of Functional GC Activity

Urinary free cortisol (UFF) measurement in 24-h urines has been advocated as the most reliable, practical assessment of cortisol secretion [10, 92]. An early study examining urinary free cortisol (UFF) excretions in 2 to 17 years old children did not find any variation with age after the 24-h excretion data had been corrected for BSA [82]. These findings are not inconsistent with the increase in cortisol secretion as determined by GC3/BSA in our 12-14 years old adolescents (Fig. **5**). In contrast to what – for practical considerations – is often oversimplified that UFF reflects adrenocortical activity or adrenal cortisol secretion, UFF does this primarily not [93, 94]. It rather reflects plasma free cortisol concentrations, as is explained below.

That both cortisol secretion (GC3/BSA) and the plasma free cortisol marker UFF/BSA actually drift apart in normal children > 10 years of age, has been analyzed by us in the same groups of healthy individuals [95] and the corresponding data are shown in Fig. **5** and Fig. **6**. Our 24-h UFF measurements depicted in Fig. **6** confirm the earlier work of Gomez *et al.* [82] not only with respect to age independence of BSA-corrected UFF values, but also with respect to the age-dependent decline in the corresponding UFF/creatinine ratios.

In a recent paper, Legro *et al.* reported increasing levels of UFF/BSA and UFF/creatinine in adolescent Caucasian females from age 12 years onwards [96]. These findings are in contrast with the results of Gomez *et al.* [82], and would imply that serum free cortisol possibly increases during puberty. Unfortunately, the authors applied different "established radioimmunoassay (RIA) methods" in their study. One problem of many commercial immunoassays for cortisol is that they have considerable cross-reactivities with other, partly unidentified, but quantitatively relevant urine steroid metabolites [97-99]. Legro *et al.* found increases for UFF/

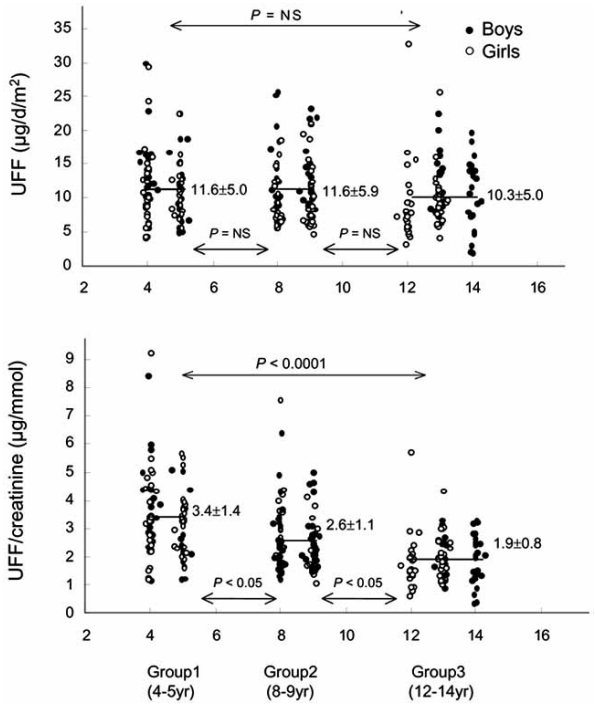

Fig. (6). Daily urinary free cortisol excretion (absolute and creatinine-corrected) in 300 healthy children [95].

BSA from 35 µg/m^2/24h in 12 year old girls to around 50 µg/m^2/24h in the 14 years old [96]. We measured constant BSA-corrected UFF excretion values of around 11 µg/m^2/24h using a non-commercial highly specific radioimmunoassay [100] after sample extraction with dichloromethane and chromatographical purification with Celite columns [95]. Mean 24-h UFF/BSA excretion rates exceeding 20 - 30 µg/m^2/24h in healthy children, adolescents are indicative of underlying substantial cross-reactivities of the assays (for explanation see section K). However, irrespective of these analytical methodological problems, the study of Legro *et al.* is the first, examining UFF excretion longitudinally during perimenarche which in principle allows the most sensitive analyses of moderate endocrine changes. Thus, their paper adds substantially to the growing evidence that important changes in GC metabolism occur or start during puberty. Unfortunately, our cross-sectional examinations showing constant UFF/BSA (Fig. **6**) and urinary free cortisone (UFE)/BSA (see legend of Fig. **7**) excretions in healthy children only reached until age 13 in girls and age 14 in boys, while Legro *et al.* examined the perimenarchal age range from 12 to 17 years.

Given a mean rate of cortisol secretion of 10 mg/d [10], the amount filtered by the kidney and excreted as UFF (< 100 µg) is less than 1 % [10]. Irrespectively, of whether UFF is mainly a product of glomerular ultrafiltration of plasma free cortisol [93] or whether it may also originate in relevant proportions from intra-renal tissue concentrations [17], it is widely accepted that its measurement in 24-h urine samples grossly integrates (as an index) the plasma free cortisol concentrations during the entire day [10]. Accordingly, instead of using the term cortisol secretion, an alternative more gen-

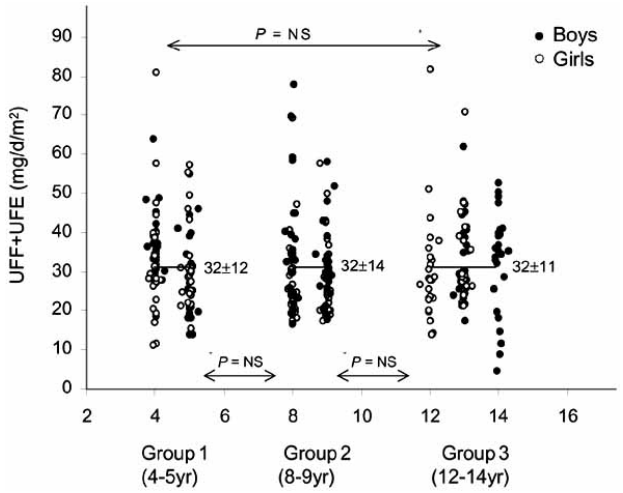

Fig. (7). Potential bioactive free glucocorticoid excretion (sum of urinary free cortisol (UFF) and urinary free cortisone (UFE)) in 24-h urine samples of 300 healthy children after body surface area (BSA) correction. UFF/BSA and UFE/BSA alone showed age independency too.

eral phrasing for what UFF stands for, could be, that UFF measurement (like salivary cortisol) is an index for the biological active fraction of cortisol in normal subjects and patients, e.g., patient with Cushing's disease [12]. However, it should already be mentioned here that the sole use of UFF as non-invasive urine marker of a subjects' functional cortisol activity can be misleading since a varying 11b-HSD2 activity, with correspondingly varying urinary free cortisone, may confound the measurement (see section G).

The fact that variation of cortisol production explains around 50 % of the variability of plasma free cortisol concentration [101] indicates that plasma free cortisol and probably also UFF are considerably affected by other factors too. However, if peripheral and/or hepatic cortisol clearance is accelerated, for example with increasing body fatness, the closely HP axis-controlled bioavailable cortisol fraction, i.e., plasma free cortisol, remains largely unchanged due to an accordingly adjusted acceleration in cortisol production. [101]. Conveying these observations to the increase in GC3/BSA (i.e., GC secretion) in our 12 -14 years old children (Fig. **5**) which was not accompanied by a parallel increase in UFF/BSA (Fig. **6**), leads to the idea that an altered clearance of cortisol may also be present during puberty (in the 12 -14 years old children). This change in clearance may have caused the observed increase in GC secretion (GC3/BSA) to assure a constant bioactive cortisol level (UFF/BSA) from childhood onwards. Actually, Charmandari *et al.* [102] observed an increased clearance of total and free serum cortisol in pubertal compared to prepubertal patients with congenital adrenal hyperplasia. However, the underlying mechanisms is still a matter of speculation, particularly in healthy, normally growing children.

A recent paper reported that both cortisol production and plasma free cortisol is increased in elderly compared to younger adults [101], However no corresponding increases, but essentially constant 24-h UFF excretion rates have been observed with normal aging in 3 other studies [103-105]. Since both UFF and blood free cortisol are thought to reflect the bioavailable fraction of cortisol, rather parallel changes in UFF and circulating free cortisol would have been expected in the elderly. Yet, such seemingly divergent findings on UFF and blood free cortisol are not necessarily inconsistent, because without simultaneous consideration of circulating and renally excreted cortisone, only a part of the potentially bioactive glucocorticoid side is taken into account. Overall, evidence grows, that in a number of physiological and pathophysiological conditions the sole look on cortisol may be insufficient to assess glucocorticoid status appropriately.

G. Role of Urinary Free Cortisone and 11b-HSD2 in the Non-Invasive Assessment of Functional GC Activity

More than one decade ago, Friedman and Yanovski [60] reported considerable overlap not only of plasma cortisol parameters, but also of 24-h UFF between patients with confirmed Cushing syndrome, patients with pseudo-Cushing states, and healthy controls precluding each of the measurements alone from being a specific diagnostic tool. Since then a growing number of researchers have suggested that the simultaneous measurements of more than one analyte in the same sample material will allow to bring more confidence to the acceptance or rejection of specific diagnoses or conclusions [64, 106-108]. In this respect, Lin *et al.* [106] found clear advantages in the diagnosis of Cushing's syndrome after simultaneous measurements in 24-h urine samples of UFF and UFE compared to UFF measurement alone.

Considering the relatively high level of free circulating cortisone in blood, which at the evening corresponds closely to free plasma cortisol concentration of ≈ 10 nmol/L [8], it becomes clear that additional data on cortisone (apart from cortisol) may allow a more specific evaluation of the overall potentially bioactive glucocorticoid fraction. Especially the fact that the low plasma protein binding of cortisone assures an ample supply of this reversibly deactivated substrate for target tissue-specific reactivation to cortisol by 11b-HSD1 [8], underlines the glucocorticoid potency of cortisone. Its local conversion to cortisol appears to be also essential in the hypothalamus and the pituitary for a normally functioning feedback regulation of the entire HPA axis [23]. Due to the clearly different serum protein binding properties of cortisol and cortisone [8, 109], the ratio of both GCs determined in blood can not provide appropriate information on specific 11b-HSD activities [16, 17]. The different clearance rates of both steroids that result from altered CBG and albumin bindings are reflected in markedly lower variations of plasma cortisone compared to plasma cortisol after insulin or ACTH infusion in healthy volunteers [4]. Although these results exclude reasonable estimations of activities of either the cortisol regenerating 11b-HSD1 or the cortisol inactivating 11b-HSD2 in blood samples, corresponding estimates are regularly calculated from urine measurements.

In line with the physiological importance of the prehormone cortisone is the fact that its renal excretion in the unconjugated free form (UFE) is about twice as high as the renal excretion of UFF [16, 93, 95, 106]. According to Best and Walker [17] this comparably high urinary excretion of

free cortisone reflects its intra-renal concentration, resulting from the particularly high expression of the cortisol inactivating 11b-HSD2 isoenzyme in the kidney. This and the fact that plasma cortisone falls to values 1/10 of the normal in patients who have undergone bilateral nephrectomy [16] confirms earlier studies that the kidney is the principal source of cortisone in man [110]. Accordingly, measurements of UFF and UFE together in the same urine samples appear to allow a relative specific assessment of renal 11b-HSD2 [16, 17, 20, 111-113].

Some authors prefer to estimate 11b-HSD2 from the ratio of the urinary excretions of the 3 major GC metabolites, i.e., from (THF+5a-THF)/THE [70, 114], however, as is explained in section I, this ratio is rather an index of the overall activity of 11b-HSD1 (without allowing to differentiate between the particular contributions of liver and fat tissue). An additional strong argument against the use of these 3 major metabolites for assessment of the activity of the isoenzyme type 2 is that the bulk of all 3 GC degradation products, i.e., about 90 %, is excreted as glucuronides [17] and most of the glucuronidation takes place in the liver, where type 1 and not type 2 is active. Recently, Ferrari *et al.* [115] re-examined both the UFF/UFE and the (THF+5a-THF)/THE ratio after glycyrrhetinic acid ingestion in healthy volunteers and suggested from the observed increases in both ratios and from higher intra-individual variations for UFF/UFE that the later ratio does not appear to be more sensitive than (THF+5a-THF)/THE. However, the changes reported by Ferrari *et al.* corresponded closely to those determined earlier in the basic studies of Palermo *et al.* [16] and Best and Walker [17]. In all 3 papers almost 1.8 fold increases were determined for (THF+5a-THF)/THE after glycyrrhetinic acid ingestion. These consistent data again underscore the overall quantitative importance of cortisone production through cortisol inactivation by the kidney which, if inhibited, delivers markedly elevated amounts of cortisol to the liver resulting in a subsequently higher relative glucuronidation of THFs compared to THE. The glycyrrhetinic acid-induced rises of UFF/UFE, however, were in all 3 studies at least 3fold, demonstrating the more accurate reflection of renal 11b-HSD2 inhibition by the ratio of the unconjugated hormones.

With regard to an appropriate assessment of 11b-HSD2 activity, not only the use of the (THF+5a-THF)/THE ratio would lead to comparatively unspecific or inaccurate results, additional pitfalls exist. Recently Quinkler *et al.* [24] directly analysed 11b-HSD2 mRNA expression in kidney biopsies of patients with various suspected renal diseases and compared the expression results with measurements of UFF/UFE and (THF+5a-THF)/THE. The authors did not find a significantly better association of mRNA expression with UFF/UFE than with (THF+5a-THF)/THE. They measured all steroid analytes by gas chromatography/mass spectrometry (GC-MS) which, if only one conventional measurement run (including enzymatic conjugate hydrolysis) is performed, generally results in quantification of the so called total cortisol and total cortisone fractions consisting primarily of conjugated cortisol and cortisone, respectively with only little amounts of the free steroids [17, 72]. Since conjugation is primarily located in the liver, an agreement of both correlations, namely "total cortisol/total cortisone" with renal 11b-HSD2 mRNA expression and (THF+5a-THF)/THE (glucuronidation rate of about 90 %) with 11b-HSD2 mRNA expression would not be striking. Unfortunately, Quinkler *et al.* did not clearly explain whether they used methodologically different GC-MS procedures to analyze UFF and UFE separately from THF, 5a-THF, and THE. What can be inferred from this issue, is that the examination of the ratio of total (conjugated and free) cortisol to total (conjugated and free) cortisone – as would be obtained by usual urinary steroid GC-MS analysis praxis – will probably not provide the optimal approach for the assessment of renal 11b-HSD2.

Since 11b-HSD2 is such an important enzyme that can considerably alter the body's cortisone production through varying cortisol inactivation and since renal excretion of cortisone – in the unconjugated free form (UFE) – is about twice as high as the renal excretion of UFF [16, 93, 95, 106], it appears to be physiologically plausible to measure not only the unconjugated 11b-OH form. However, UFE measurements are not regularly performed. In the literature mean 24-h excretion rates of about 55 to 73 µg/d [16, 17, 106] for adults and 15 to 24 µg/d [16, 95] for children have been reported. Taylor *et al.* [93] have recently published reference intervals for UFE that cover the aforementioned average values for children and adults. A few years ago, we checked whether daily UFE excretion in children would show the same age-independent pattern during growth as is present for UFF after the data are corrected for individual BSA [95]. As with UFF/BSA, also no variation with age was seen for UFE/BSA indicating that in healthy children no marked age-dependent influence on renal 11b-HSD2 exists that may alter urinary free GC excretion rates.

Potentially Bioactive Free GCs

Accordingly, when calculating the ratio of UFF to UFE as the metabolomic expression of 11b-HSD2 [95] and the sum of UFF + UFE (both BSA-corrected) as a new combined measurement to cover both potentially bioactive free GCs (Fig. **7**) no significant changes were discernible with age. Since foods like liquorice (*via* its glycyrrhetinic acid content) and probably other environmental factors too, can markedly influence the kidney's 11b-HSD2 – which would confound UFF measurements resulting in reciprocal changes of UFF and UFE – we hypothesised that the sum of both free steroids may reflect the *in vivo* bioactive GC level better than UFF alone and we termed the UFF+UFE sum potentially bioactive free GCs [64]. This approach is strongly supported by the basic results of Best and Walker [17] who – after administering several combinations of 11b-HSD2-affecting drugs to healthy men – measured marked changes in 24-h UFF, 24-h UFE, and in the daily GC secretion (24-h C21) necessary to maintain an appropriate, probably constant bioavailable GC level in circulation. What actually remained fairly constant in these authors' study, was the sum of UFF and UFE despite the clear drug effects on 11b-HSDs [17]. Additional strong evidence was provided in a recent study on children with type 1 diabetes, in which the existing increased GC bioactivity could not be proved by UFF, but by UFF+UFE measurements [64].

These results together with the clear advantages reported by Lin *et al.* [106] for the diagnosis of Cushing's syndrome

after simultaneous measurements of UFF and UFE, strongly suggest that cortisone, quantified in its urinary free form, essentially complements UFF as a second potentially bioactive GC metabolite, in all physiological and pathophysiological conditions in which alterations in 11b-HSD2 activity can not be excluded.

H. A Role of Urinary 6b-Hydroxycortisol for Non-Invasive Assessment of Functional Glucocorticoid Activity?

Cortisol is metabolised by reduction of the A ring of the steroid molecule and reduction of the 20-ketone group or by 6 beta-hydroxylation. The latter step leads to the formation of 6beta-hydroxycortisol (6b-OHF) primarily located in the liver. 6b-OHF is excreted in urine by the kidney as a polar unconjugated molecule and represents 1 % of the total cortisol metabolites in urine [116]. Transformation of cortisol to 6b-OHF is catalysed by cytochrome p450 3A4 [116, 117], a drug- or xenobiotic-metabolising enzyme system and measurement of urinary 6b-OHF excretion in relation to UFF is frequently used as a non-invasive test to assess induction or inhibition of this enzyme's activity [118-120]. A detailed overview on the potential and limitations of this kind of *in vivo* probe has recently provided by Galteau and Shamsa [116].

Since the early sixties it was known that 6b-OHF, which is also a minor secretion product of the adrenal gland [3], is renally excreted in considerably elevated amounts in patients with Cushing's syndrome [121]. In 1979, Voccia *et al.* [122] provided evidence that measurement of urinary 6b-OHF may be preferred diagnostic test for identification of several hypercortisolemic states. Also plasma and salivary 6b-OHF determinations have been advocated to precisely detect not only overt increases of cortisol secretion but also mild GC overproduction [63]. A peculiarity of 6b-OHF is its considerable polar character resulting in an almost exclusive excretion as an unconjugated molecule by the kidney.

Saenger, in 1983, was the first to demonstrate that 6b-OHF excretion paralleled UFF excretion and was highest between 08:00 and 12:00 and lowest around midnight, in principle reflecting the changes in adrenal secretion [123]. Parallel diurnal rhythms of urinary 6b-OHF and UFF have also been described several years later [124], confirming the close link of both forms of cortisol. All together, the published findings suggest that 6b-OHF is one of the direct metabolic products (primarily 6beta-hydroxylated in the liver) of bioavailable free GCs in blood, implying a previous intra-hepatic 11-keto reduction of the cortisone taken up from the circulation. If it is correct that (apart from a hepatic enzyme induction through certain drugs and other exogenously ingested components) 6b-OHF would really reflect hyper- and hypocortisolemic states, i.e., the bioavailable free GCs, then this water soluble metabolite should show the same excretion profile as UFF+UFE in normally growing children not using drugs. Actually, after examining urinary 6b-OHF by GC-MS in 300 healthy children and adolescents a fairly age-independent excretion pattern has been observed by us for this steroid after correcting its 24-h excretion values for BSA (Fig. **8**). Absolute daily excretions of our 16-18 years old corresponded closely to the reference values reported by Lee *et al.* for adults as measured by reversed-phase high performance liquid chromatography [124]. These data demonstrate that, apart from UFF+UFE, the GC metabolite 6b-OHF may be an alternative non-invasive marker for the functional cortisol activity if drug ingestion can be excluded. However, it should be kept in mind that additional confounders may alter hepatic cytochrome p450 3A4 activity and thus 6b-OHF production, for example, hyperthyroidism [65] and other illnesses [117, 120] as well as heavy metal intoxications [125]. Also conditions with strong changes in faecal production or biliary secretion might result in altered urinary 6b-OHF excretions since, contrary to most other GC metabolites, 6b-OHF seems to be excreted predominantly (2/3) in the faeces [126]. Furthermore, data on the possible impact of renal impairment on 6b-OHF are lacking.

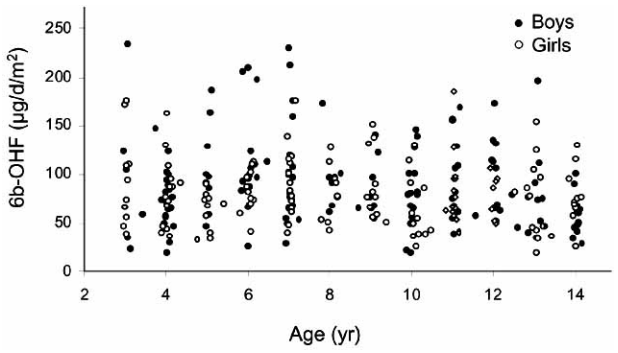

Fig. (8). 24-h urinary 6beta-hydroxycortisol excretion rates in 300 healthy children aged 3 to 14 years. The study is described in detail in reference [132].

Our findings of quite constant urinary excretion rates of 6b-OHF/BSA (Fig. **8**), (UFF+UFE)/BSA (Fig. **7**), and UFF/BSA (Fig. **6**) in healthy children during growth agree with the UFF/BSA pattern as published by Gomez *et al.* [82] and strongly suggest that the bioavailable, potentially bioactive fraction of cortisol and cortisone in the circulation of children may not vary considerably during growth. Despite – to our knowledge – lacking confirmative data that plasma free cortisol is actually constant from childhood to young adulthood, it appears that the pubertal increase in cortisol secretion observable around 12-14 years (GC3/BSA, (Fig. **5**), is essential to ensure this postulated constant functional systemic glucocorticoid activity. The necessity for such an increase in GC secretion during puberty has already been suggested by Charmandari *et al.* [102] who observed a rise in serum free cortisol clearance in pubertal patients with classic congenital adrenal hyperplasia. However, it is not yet clear which of the glucocorticoid metabolising enzymes are primarily responsible for the elevated cortisol degradation. Thus, ongoing research in 24-h urine samples of healthy children will help to identify relevant global enzyme activities involved.

I. Urinary Metabolite Ratios to Assess Global Steroid Metabolizing Enzyme Activities

Apart from examining the kidney's 11b-HSD2 activity marker UFF/UFE, further enzyme activity indices are regularly checked non-invasively. A frequently used *in vivo* in-

dex of "global" 11b-HSD1 activity is the urinary ratio of A-ring reduced cortisol metabolites (THF and 5a-THF) to the tetrahydro-metabolite of cortisone (THE), with an increased (THF+5a-THF)/THE ratio indicative of an increased enzyme activity. However, this index is clearly confounded by renal 11b-HSD2 activity, in that increases of (THF+5a-THF)/THE are also discernible if a reduced 11b-HSD2 activity, indicated by an elevated UFF/UFE, delivers surplus cortisol from the kidney to the liver (for a raised hepatic i) cortisol → THF and ii) cortisol → 5a-THF conversion). To assure that a change in the (THF+5a-THF)/THE ratio is highly suggestive of an isolated effect on "global" 11b-HSD1 activity, an unaltered UFF/UFE ratio has to be demonstrated [22]. Thus, measurements of both ratios (THF+5a-THF)/THE and UFF/UFE allow to specify whether the type1 enzyme activity may have been increased or decreased globally. However, due to the fact that 11b-HSD1 is not only expressed in the liver, but also markedly in fat tissue, it is at present, not possible to specifically allocate an activity change to a particular organ or tissue. Also, increases in one tissue and simultaneous decreases in the other are possible which is difficult to unravel at present.

Another frequently determined global enzyme activity indicator, which is also based on non-invasive urinary GC metabolite measurements, assesses overall 5a-reductase (5a-R) activity. For this, the ratio of urinary 5a-THF to THF (or the reciprocal term) is usually examined [31, 65, 69, 72, 74, 127]. 5a-R is a steroidogenic enzyme responsible for both 5a-reduction of cortisol to 5a-dihydrocortisol predominantly in liver and testosterone to 5a-dihydrotestosterone predominantly in skin [69]. 5a-Reduction is essentially irreversible and flattens the steroid molecule due to an altered relation of the A and B rings. This irreversible molecule flattening either inactivates (cortisol) or activates (testosterone) the steroid hormones.

The conventionally calculated 5a-THF/THF ratio reflects in the first instance the balance between 5a-R und 5beta-reductase activities in liver and other tissues including fat [74, 128]. Increases in this ratio could therefore imply a decrease in 5beta-reductase or an increase in 5a-Red. If however, the 5beta metabolites THF and THE are not reduced, then changes in the 5a-THF/THF ratio represent an index of 5a-Red activity. Studies examining this ratio in healthy young adults suggest that a sex difference exists with a relatively higher 5a-reduction in males than in females [73, 129]. Hints for an increased 5a-reduction were also observed in obese adults [31, 74] indicating that cortisol clearance may be enhanced in subjects with higher body fat [130]. This may explain at least in part the frequently observed increase in HPA axis activity in obesity [31-34]. First evidence for a probably divergent association of the 5a-THF to THF ratio with visceral fat on the one hand and liver fat on the other hand has been provided by Westerbacka *et al.* [128]. While this ratio was high in subjects with high visceral and low liver fat, it was lowest in volunteers showing low visceral and high liver fat. High ratios of 5a-THF to THF in the urine, being consistent with enhanced 5a-R activity, are also regularly seen in patient with polycystic ovary syndrome [69, 72, 127] and recently Goodarzi *et al.* provided genetic evidence for a relevant role of 5a-R isoenzymes in the pathogenesis of PCOS [131]. All these studies suggest that urinary analyses of THF and 5a-THF may provide a useful means to non-invasively assess global 5a-R.

A number of additional urinary metabolite ratios can be used to grossly assess particular enzyme activities of human steroid metabolism, e.g., 3b-hydroxsteroid dehydrogenase, 17b- hydroxsteroid dehydrogenase or 21-hydroxylase activity [132]. Recently we could show in healthy children and adolescents [132] that the urinary steroid metabolites, commonly used for the evaluation of possible 21-hydroxylase deficiency, did not show the same variation with increasing age as published data on immunohistochemically determined 21-hydroxylase activities in normal human adrenal glands. However, we identified a particular precursor/product ratio of which the metabolites differed only in the hydroxy group in position C21 (5β-pregnane-3α,17α,20α-triol-11-one / 5β-pregnane-3α,17α,20α,21-tetrol-11-one), that showed a closer association with the immunohistochemical findings [132]. Whether this new steroid metabolite ratio may allow a more sensitive evaluation of 21-hydroxylase activity than the commonly used indices for monitoring treatment in 21-hydroxylase deficiency, has not been tested yet.

J. Concurrent Assessment of Glucocorticoid Secretion, Functional Glucocorticoid Activity, and Underlying Glucocorticoid Metabolism in a Single 24-h Urine Sample

As has been explained in detail, i) measurement and summation of the major GC metabolites of 24-h urine samples (C21 or GC3, for definition see section A) provide a time-integrated, stress-free, in-vivo result of the amount of cortisol and cortisone originally secreted by the adrenal gland, ii) quantification of the sum of UFF and UFE yields an index for the biologically active (free) fraction of circulating cortisol and cortisone, and iii) determination of certain urinary steroid metabolite ratios allows the assessment of particular global steroid metabolizing enzyme activities. Thus, it is in principle possible to non-invasively characterize the overall GC status of children, normal adults, and patients by specific steroid hormone analyses in 24-h urine samples.

Examples of this are the syndrome of apparent mineralocorticoid excess (AME), very low calorie diets, starvation, hyperthyroidism, or Type1 diabetes mellitus.

Apparent Mineralocorticoid Excess

In the syndrome of AME, mutations in the gene encoding 11b-HSD2 account for an absent or low inactivation of cortisol to cortisone in kidney, colon, and salivary gland resulting in an inherited form of hypertension and hypokalemia. GC secretion rate often falls to very low levels due to the prolonged plasma half-life of cortisol and a normal intact negative feedback mechanism [21]. (i) Accordingly, urine analysis shows a decrease in the total urinary excretion of cortisol metabolites (C21) [21]. (ii) Since UFE is almost undetectable, UFF alone, but not UFF+UFE is elevated in this illness [16, 21], indicating an increased functional cortisol activity at least in the kidney. It should be mentioned that clear UFF elevations are probably no longer detectable if renal function becomes impaired in the course of the illness [133, 134]. (iii) The elevated renal cortisol delivery to the liver is one reason

for the simultaneously elevated (THF+5a-THF)/THE ratio [16, 21, 135], which, in this case of unusually high UFF/UFE, rather reflects an index of global 11b-HSD activity than of hepatic or adipose tissue 11b-HSD1 activity [21]. Other urinary cortisone metabolites such as THE and cortolones are also greatly diminished due to 11b-HSD2 deficiency. In addition, urinary 5a-reduced cortisol metabolites (e.g., 5a-THF) predominate over 5b-reduced cortisol metabolites (THF), consistent with a relative increase of 5a-reductase over 5beta-reductase activity, which probably reflects the liver's contribution to an accelerated degradation of the elevated cortisol level.

Very Low Calorie Diet

(i) Three weeks on a very low calorie diet (VLCD) with 10 % weight loss results in a significantly reduced urinary excretion of all principal cortisol metabolites (C21) [136] demonstrating a reduced adrenal GC secretion. (ii) The urinary free cortisol/cortisone ratio was found to be normal in the study by Johnstone *et al.*, which hints at a normal UFF+UFE level during dietary restriction, but unfortunately the corresponding absolute 24-h excretion data were not presented in that paper [136]. The observed normal plasma CBG and normal plasma cortisol concentrations in VLCD subjects [136] would be in line with normal absolute UFF and UFE levels. (iii) Apart from an unchanged (THF+5a-THF)/THE ratio, reflecting usual 11HSD1 activity, the restricted diet has induced a fall in the ratio of 5a-THF to other cortisol metabolites, i.e., a fall in 5a-reductase activity [136], which suggests a slowing in cortisol catabolism. The resulting increase in cortisol half-life could explain the observed decrease in GC secretion (C21), since less newly secreted cortisol from the adrenal gland is necessary to maintain normal circulating cortisol.

Starvation

(i) During starvation at 5 % weight loss, urinary C21 remains mostly unaltered [136]. (ii) The urinary free cortisol/cortisone ratio is increased suggesting an increased functional cortisol activity (UFF and UFE excretions were unfortunately not provided) [136]. In line with such an increased stress level, total plasma cortisol is markedly elevated both in the morning and evening compared with pre-starvation values whereas CBG remains essentially unchanged. (iii) Similar to the VLCD, a urinary marker of 5a-reductase activity (5a-THF/THF) decreases in starvation. Both this probable decrease in cortisol degradation and the increase in the free cortisol/cortisone ratio (increased renal cortisol release) could explain why short-term starvation emerges as a stress condition with functionally elevated GC activity, but without necessarily elevated adrenocortical activity.

Hyperthyroidism

(i) Total urinary cortisol metabolite excretion and thus adrenocortical activity (adrenal GC secretion) is markedly increased in hyperthyroid patients [65]. (ii) Also the sum of UFF and UFE is increased, with a preponderance of UFE over UFF [65, 137]. However, this increase in functional GC activity remains masked if only UFF is measured, since 24-h UFF excretion rates are normal [65]. (iii) Due to the increased renal cortisone production, THE is also elevated and the (THF+5a-THF)/THE ratio is decreased [65]. Elevated 5a-THF and normal THF excretion rates indicate a considerably increased 5a-reductase activity and thus a reduction in cortisol half-life [65]. This stimulated cortisol degradation can explain why the adrenal gland's activity is so clearly increased, i.e. why a markedly GC hyper-secretion exists, although functional GC activity (UFF+UFE) appears to be only moderately elevated.

Type1 Diabetes Mellitus (T1DM)

(i) Children with T1DM show mostly normal urinary C21 excretion rates [64]. (ii) As in hyperthyroidism, the 24-h UFF excretion rates are unchanged and those of UFE are elevated [64]. The increased UFF+UFE indicates the existence of a stress condition despite normal adrenal GC secretion. (iii) Markedly reduced 5a-THF/THF ratios strongly suggest slowed metabolic clearance of cortisol [64], which probably contributes to higher circulating bioavailable GC concentrations without additional adrenal activation. Importantly, UFF and C21 measurements alone would leave the functional stress activity of T1DM children undetected.

The fact that some studies have reported increased UFF excretion rates in children with T1DM [138] does not contradict with the findings presented here [64], as will be briefly explained in the final section.

K. Pitfalls in Urinary Glucocorticoid Metabolite Measurements

A number of pitfalls exist when assessing GC status and measuring GC metabolites in 24-h urine samples. Relevant potential errors and potential confounders, some of which have been discussed in greater detail in the previous sections, are summarised below.

Urinary Free Glucocorticoids and Renal Function

As has been convincingly shown for UFF, its 24-h excretion rate is significantly reduced in subjects with renal impairment (creatinine clearance < 60 mL/min) [133, 134]. This diminished UFF output can be quite accurately predicted by increased serum creatinine concentrations. If adequacy of kidney function is unknown, a low UFF (and probably also a low UFF+UFE) may be falsely interpreted as diminished functional GC activity. Thus, an important prerequisite for a meaningful interpretation of UFF measurements in diagnostics and research, seems to be that kidney function is normal or at most only modestly impaired. The influence of an impaired renal function on measurements of UFE and other urinary steroids and analytes, such as GC3, C21, androgenic C19 steroids or catecholamines needs to be clarified [134]. Also the recently reported influence of diuresis and water loading on UFE and UFF excretion rates indicate that further confounders of urinary free glucocorticoid measurements may exist [139]. Accordingly, future studies on the relevance of variation of hydration status for assessment of functional GC status appear necessary.

Urinary Free Cortisol and Immunoassay Cross-Reactivities

Analysis of UFF and UFE in a complex matrix such as urine is a challenge for the laboratory [93]. Values of UFF for certain groups of patients vary widely in the literature

[98], and vary also from immunoassay kit to kit [140]. Most commercial immunoassays overestimate true UFF, and this overestimation can be as high as threefold due to considerable cross-reactions with unknown [98] and known metabolites. Among the cross-reacting metabolites, synthetic substances as well as physiological urine steroids have been identified. Fink *et al.* [141] reported the test results of one kit with a 100 % increase over placebo values for 24-h UFF excretion after administration of a synthetic GC (budesonide) that definitely suppressed endogenous GC secretion. Urinary C21 fell by 24 % [141]. Horie *et al.* [140] tested 4 different immunoassays, and found clear cross-reaction with 5a-THF in 3 commercially available kits, implying that the use of such assays would not allow appropriate differentiation between GC secretion and functional GC activity, the levels of which can markedly diverge, even in opposite directions. Lee and Goeger [99] found 6b-OHF to be a significant source of interference in UFF immunoassays, which would explain why in some studies on children with Type1 diabetes mellitus [138], increased UFF excretion rates have been observed, while other examinations did not find a consistent increase in 24-h excretion of C21 (GC secretion) and UFF, but specific strong elevations in urinary 6b-OHF [64]. Both for physiological research and for appropriate diagnosis and characterisation of functional glucocorticoid activity in patients (e.g., with preclinical Cushing's syndrome), accurate and precise UFF measurements are definitely required. A simple and inexpensive solid-phase-extraction prior to immunoassay analysis (or other chromatographical purification steps) could considerably improve accuracy and precision [99]. Taylor *et al.* provided reference intervals for UFF and UFE excretion rates which suggest that – as a rule of thumb – mean daily UFF and UFE excretion of groups of normal subjects should not exceed 60 µg/d (females 43 µg/d) and 141 µg/d (females 122 µg/d), respectively [93].

Tetrahydrocortisone and Tetrahydrocortisol Metabolites and 11b-HSD2

If adequate UFF and UFE analyses are obtained and renal impairment can be ruled out for the urine samples under study, the calculation of the UFE/UFF ratio provides a more sensitive index of renal 11b-HDS2 activity than the (THF+ 5a-THF)/THE ratio [17]. For further details see section G. Despite the fact that the tetrahydro-metabolites of the latter ratio are primarily formed extra-renally and are conjugated mainly in the liver, several authors prefer to assess the kidney's 11b-HSD2 activity by using this ratio. In principle however, this ratio reflects the total balance of both 11b-HSD isoenzymes (Type 1 and 2).

Tetrahydrocortisone and Tetrahydrocortisol Metabolites and 11b-HSD1

Much more frequent than its usage as an index of 11b-HSD2, is the usage in the literature of the (THF+5a-THF)/ THE ratio as an index of 11b-HSD1. The (THF+5a-THF)/THE ratio is clearly confounded by renal 11b-HSD2 activity, in that an increase in (THF+5a-THF)/THE occurs for example, if reduced 11b-HSD2 activity, indicated by a suppressed UFE/UFF, delivers an excess of non-inactivated cortisol from the kidney to the liver. In the liver, the raised cortisol flux is dealt with by an elevated conversion of cortisol to THF and to 5a-THF resulting in the (THF+5a-THF)/THE rise. Accordingly, the latter ratio is primarily an unspecific index for the total balance of both 11b-HSDs, as already mentioned above. However, if the activity of 11b-HSD2 is controlled for, and an unaltered UFE/UFF ratio is confirmed by appropriate urine measurements, then urinary (THF+5a-THF)/THE indicates global 11b-HSD1 activity, i.e., cortisol reactivation from circulating cortisone in liver and adipose tissue (For further details see sections G and I).

Urinary Free Cortisol and Glucocorticoid Bioactivity

Irrespective, of whether UFF is mainly a product of glomerular ultrafiltration of plasma free cortisol [93], or whether it may also originate from intra-renal tissue concentrations [17], its 24-h urine measurements have been widely used as an index that grossly integrates the plasma free cortisol concentrations over the entire day [10]. However, in the meantime there is growing evidence that a markedly varying 11b-HSD2 activity, resulting in a considerably varying kidney-related inactivation of cortisol can clearly confound UFF measurements [64, 65, 106], so that an increased stress level can no longer be definitely inferred from an elevated UFF alone (see sections G and J). Various studies suggest that UFE is an important complementary analyte to UFF in the non-invasive assessment of functional glucocorticoid activity [64, 65, 106]. Determination of the sum of both free steroids appears to be a more specific non-invasive marker of functional cortisol activity than UFF alone. In other words, sole consideration of UFF may be insufficient to assess GC bioactivity appropriately.

Measurement of Cortisol and Cortisone with Gas Chromatography/Mass Spectrometry and 11b-HSD2

If urinary cortisol and cortisone are measured by usual gas chromatography/mass spectrometry (with prior enzymatic conjugate hydrolysis) no UFF and UFE measurements are obtained. This analytical procedure yields the so-called total cortisol fraction and total cortisone fraction, consisting primarily of conjugated cortisol and cortisone respectively, with only small amounts of the free steroids [17, 72]. Since conjugation is primarily located in the liver these measurements can not be used to assess the kidneys' 11b-HSD2 activity appropriately. Total conjugated and free cortisol and cortisone are not substitutes for UFF and UFE, which have physiological implications more closely related to the kidney.

Glucocorticoids, Anthropometrics, and Creatinine

Additional pitfalls exist if urinary 24-h GC excretion rates are related to BMI instead of body surface area in children or if they are inappropriately corrected for urinary creatinine (sections D, E).

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ABBREVIATIONS

ACTH = Adrenocorticotropic hormone

AME = Apparent mineralocorticoid excess

BMI	=	Body mass index
BSA	=	Body surface area
C21	=	Total urinary cortisol metabolites
CBG	=	Corticosteroid binding globulin
CRH	=	Corticotropin-releasing hormone
FFAs	=	Free fatty acids
GC-MS	=	Gas chromatography/mass spectrometry
GC	=	Glucocorticoid
GC3	=	Sum of 5a-THF, THF, and THE
HPA	=	Hypothalamic-pituitary-adrenal
11b-HSD	=	11b-Hydroxysteroid dehydrogenase
6b-OHF	=	6b-hydroxycortisol
5a-R	=	5a-reductase
RIA	=	Radioimmunoassay
T1DM	=	Type1 diabetes mellitus
5a-THF	=	5α-tetrahydrocortisol
THF	=	5β-tetrahydrocortisol
THE	=	5β-tetrahydrocortisone
UFF	=	Urinary free cortisol
UFE	=	Urinary free cortisone
VLCD	=	Very low calorie diet

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