PRECISION ANALYTICAL INC.
CREATORS OF THE DUTCH TEST

# Clinical Validation of **DUTCH** Test

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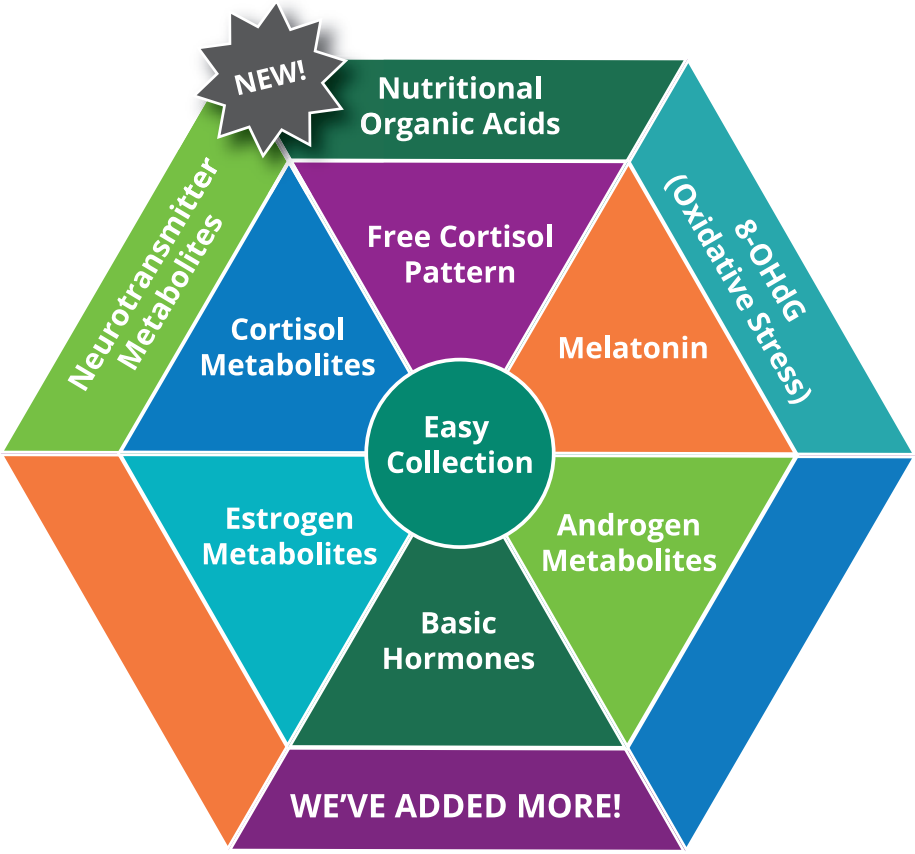
Founder and President
Precision Ananlytical, Inc.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Cortisol Metabolites
Free Cortisol Pattern
Easy Collection

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# CLINICAL VALIDATION OF DUTCH TEST

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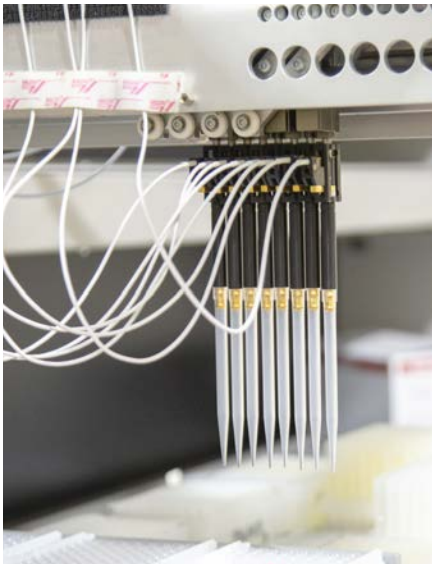
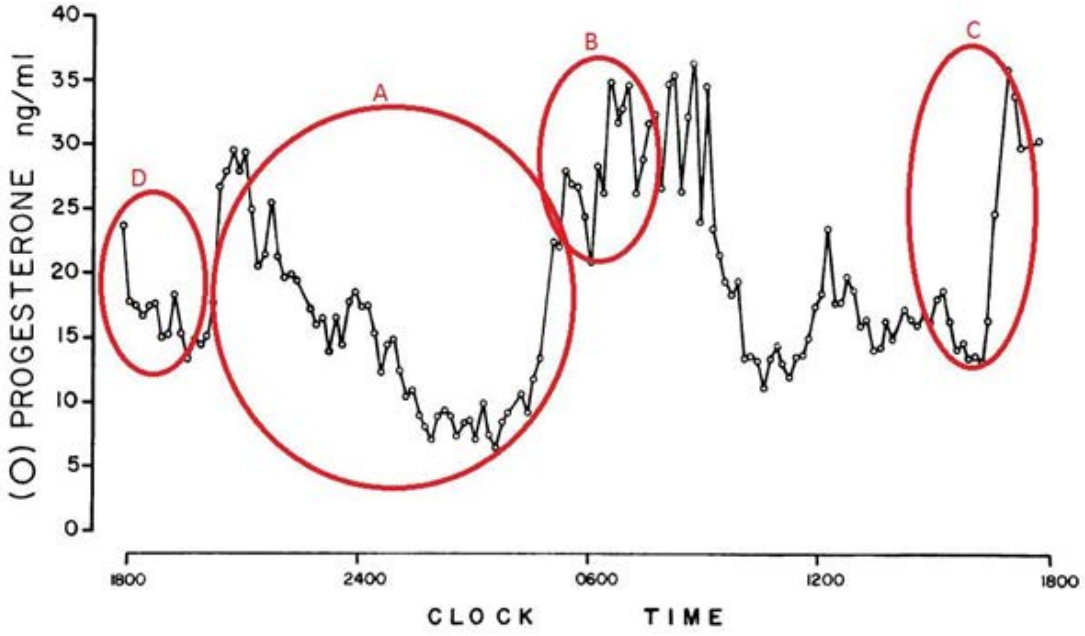
# Introduction

Given the rise in use and popularity of the DUTCH (Dried Urine Test for Comprehensive Hormones) model of testing, dialogue about the best way to test reproductive and adrenal hormones continues to advance. All lab tests have pros and cons, and DUTCH testing is no exception. Overall, it's a strong option with an impressive list of strengths and manageable caveats. Precision Analytical, Inc. maintains a transparent approach to discussing the strengths and challenges of all testing modalities. Recently, some competing labs have circulated questions about the DUTCH test. We asked ourselves many of the same questions when we began exploring the DUTCH concept. However, some of the questions raised are misleading marketing ploys to discredit a valid test. We'd like to take this opportunity to present an overview of the challenges and questions to using dried, spot--urine samples for hormone analysis, and address some of the questions regarding DUTCH and hormone testing in general.

## Have peer-reviewed journals published DUTCH data?



The scientific literature has much to say about the viability of measuring hormones in urine. We have our first peer-reviewed journal submission currently under review for publication. It will show serum and 24-hour correlation for reproductive hormones, and we can share the data as soon as the publication is formally accepted. We're excited to get our excellent validation data into peer- reviewed journals as we move forward. It is important to remember that many of the concepts used in the DUTCH test are already established in the literature. The relevance, for example, of measuring estrogen metabolites in spot urine samples is found both in the scientific literature and is offered by other labs. For this test, we are simply using dried samples, which show excellent correlation to liquid urine and great stability for months at room temperature (seen later in this document). For references, relevant to urine hormone testing, see the end of this document. One challenge to serum hormone testing is the fact that reproductive hormones change somewhat unpredictably throughout the day. Filicori, Butler and Crowley showed in the Journal of Clinical Investigation that serum progesterone values are highly variable throughout the day in premenopausal women. One advantage of the DUTCH collection and testing methodology is that the four urine samples used represent over half of the day, instead of just one moment in time. The four samples are collected to represent the four red circles below superimposed on serum data throughout the day. This woman clearly ovulated, given the level of progesterone shown; however, some measurements may imply low production (~5ng/mL) and some are rather high (>30ng/mL). The DUTCH test offers a better representation of the patient's overall progesterone production.




For each biomarker on the DUTCH test, Precision Analytical has independently validated the analytical performance (to ensure accuracy and precision) as well as the clinical viability. All hormones and metabolites have been compared favorably to 24-hour values. Reproductive hormones have been additionally correlated against serum measurements. Free cortisol values are best compared against salivary values, and you can see significant discussion of these comparisons in the rest of this document along with a comparison of actual patient samples in the appendix.

For each and every DUTCH biomarker, we are proud to have validated excellent analytical and clinical performance. Given the unparalleled comprehensiveness of the test, healthcare professionals of diverse backgrounds and specializations have found the DUTCH instrumental in their treatment of patients with a myriad of hormonal dysfunction. Below you can see the exploration of the many questions regarding the DUTCH test that we have explored over the past decade.

## Is urine free cortisol as reliable as salivary free cortisol for assessing the diurnal cortisol pattern?

This is a very important question, and it is important to carefully define both the question and the answer. Both urine and saliva have some confounding variables for testing free cortisol. For assessing only free cortisol, salivary testing does have some benefits over urine. When evaluating the literature, especially when considering the addition of the Cortisol Awakening Response (see next section for details), it is still considered the best way to test free cortisol. Be advised, this last statement is somewhat pragmatic. Testing free cortisol by methods that are not typically available (such as measuring "total" cortisol in blood along with cortisol binding globulin (CBG)) may be technically better than salivary testing. However, CBG is not a readily available test and serum is not practical to obtain at relevant times (waking, 30 minutes after waking, bedtime, etc.) Therefore, for the sake of this argument, we will consider salivary testing the gold standard for free cortisol, even though there may be an academic rebuttal to this point.

With respect to the diurnal pattern of free cortisol, the DUTCH test has proven to be a viable alternative to salivary testing, although it is a reasonable position for one to prefer saliva testing for the assessment of free cortisol. Salivary free cortisol is well supported by the literature. Measuring the diurnal pattern of free cortisol in urine is also represented in scientific literature, but not to the extent of salivary testing.

Urine cortisol testing also has some benefits compared to salivary testing. A urine sample represents more than an hour of time, which makes it less prone to transitory shifts that may not align with the overall trend of cortisol secretion. The addition of free cortisone levels, along with metabolized cortisol (not offered by serum or saliva testing), offer a broader view of cortisol production and metabolism.

The big picture offered by urine testing can skillfully point you in the direction of problems with inflammation, thyroid, blood sugar/insulin, or the HPA axis. The scales tip further in the direction of the DUTCH test when considering the wealth of information that is included (cortisone, cortisol metabolites, improved accuracy for estrogen testing, estrogen metabolites, androgen metabolites, 8-OHdG, etc.).

The patterns of free cortisol in saliva and dried urine follow the same trend. While both tests measure free cortisol, they do not represent the exact same period, which can make direct comparisons tricky. For example, a 5pm dried urine sample represents the time between the last urination and 5pm. A 5pm saliva sample taken at the same time represents just a few minutes of time. In some situations (such as at waking) it is beneficial to use a sample representing a short time period. In other situations, a saliva sample may be more prone to a transitory shift in cortisol that doesn't represent the overall cortisol pattern for the day. Over time, we have tested dozens of patients for free cortisol in both dried urine and saliva and have found good concordance between the two tests.

For a set of examples of urine and saliva results for free cortisol pattern, see the last section of this article.

## Are there scientific references in the literature for assessing the diurnal change in free cortisol using urine testing?

Yes, among others, the following are examples of published results using "spot" urine samples to assess the diurnal pattern of free cortisol. Research by Richdale and Prior1 reported using urine as a "reliable measure of cortisol excretion profile" in autistic children. They went on to report similar profiles in healthy controls, but higher levels in autistic children during school days indicating "an environmental stress response in [the autistic] group."

Contreras et al2 used spot urine samples and "clearly separated normal subjects from those with Cushing's." They did so in two different sample sets – one at bedtime (where Cushing's patients are expected to have higher results) and and one in the morning following dexamethasone suppression (where Cushing's patients are expected to have significantly higher results compared to healthy controls). They concluded that the urine collections were "a practical and simple method of assessing cortisol secretion." And "effective in assessing dynamic cortisol responses, such as diurnal variation and responsiveness to suppression."

In 2006, Jerjes3 showed proper diurnal patterns of free cortisol in healthy controls and similar, but lower patterns in patients with chronic fatigue. In a previous publication, this group showed very similar patterns in salivary analysis.

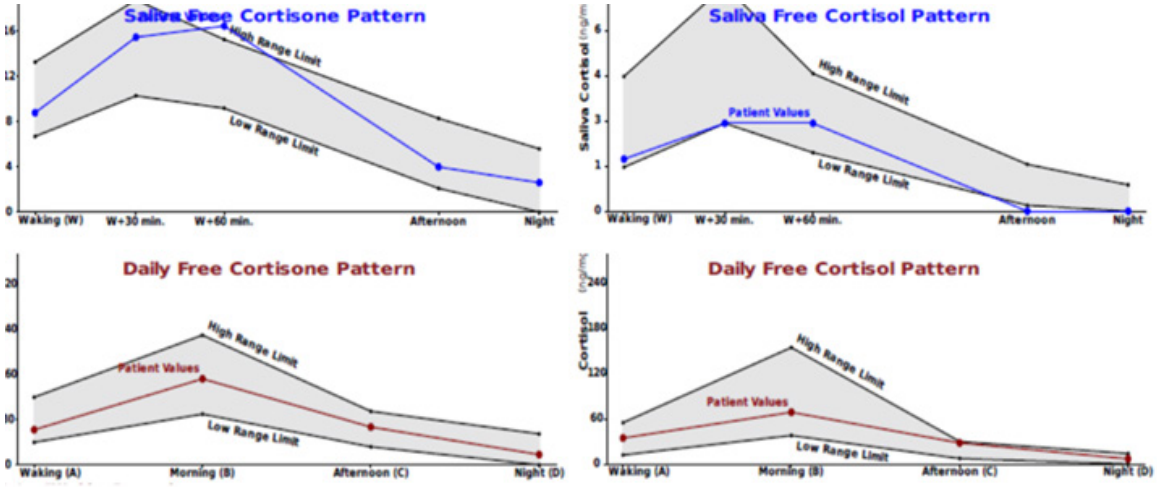
## Is there a benefit to testing free cortisone along with free cortisol?

Yes; while cortisone offers a less reliable, second version of the free cortisol pattern, measuring free cortisone really pays off in some cases. One such example is when the patient contaminates their sample with cortisol (from hydrocortisone cream). If, for example, the bedtime sample shows an odd elevation of free cortisol, the legitimacy of this elevation can be confirmed if cortisone increases as well.

As an example, the Endocrine Research Laboratory at Aurora St. Luke's Medical Center published results in which they were sent eight samples that showed very high levels of bedtime salivary cortisol. Six of the eight were shown to be contaminated. In these six samples, salivary cortisone was not elevated, and the patients were confirmed to have been taking hydrocortisone (cortisol) products that contaminated their sample. Only two of the very high bedtime cortisol measurements were legitimately from endogenous production. For these two samples, high cortisone results confirmed the state of endogenous hypercortisolism. For the other six results, the high cortisol value was misleading and clinically inaccurate. The authors suggested that LC-MS/MS analysis, which includes free cortisone, is essential for accurate clinical conclusions in these cases.4

Another benefit of testing cortisone is when patients rapidly convert cortisol to cortisone in the saliva glands. In the saliva glands, as well as in the kidneys, some cortisol is deactivated to cortisone. When this happens in excess, cortisol measurements may not tell the entire story. Having both measurements gives a fuller picture of what's going on locally and systemically. (See next page for example.)

Perogamvrosn et al make the point that salivary cortisone may be a better reflection of serum free cortisol. With hydrocortisone (cortisol) therapy or adrenal stimulation, salivary cortisone seemed to parallel serum free cortisol levels more closely than salivary cortisol. He concluded, "[salivary cortisone] closely correlated [to serum free cortisol] under all experimental conditions, being unaffected by changes in CBG and oral hydrocortisone administration, as opposed to salivary cortisol that proved unreliable in the latter setting."5 The reason he described salivary cortisol as "unreliable" with oral hydrocortisone therapy is that some samples were contaminated from the supplement. As we described earlier, cortisone comes to the rescue when samples are contaminated with cortisol.

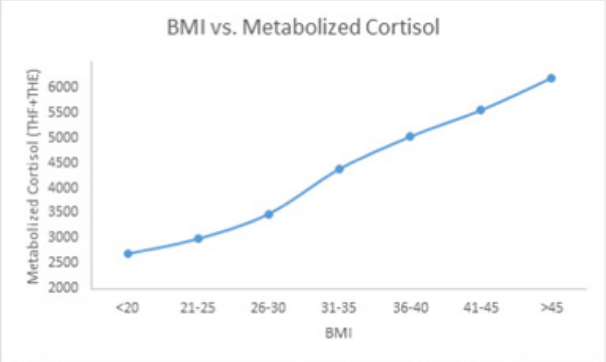


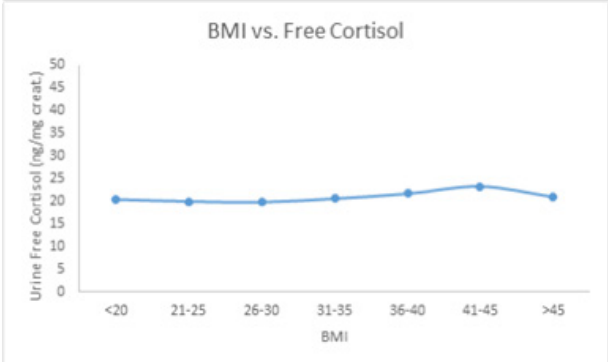

The above is an example of a patient who shows much higher cortisone levels in saliva compared to cortisol. In urine, the two show similar patterns. For some reason, this patient's cortisol to cortisone deactivation is more pronounced in the saliva gland. While salivary free cortisol says that levels are on the low side, part of that cortisol has been shuttled over to cortisone in the local tissue of the saliva gland. There is nothing wrong with testing cortisol by itself, but adding cortisone occasionally improves clinical accuracy. Adding the downstream metabolites of cortisol may add even more information to the testing (see next section).

## Is there a benefit to testing cortisol metabolism (what we call "metabolized cortisol")?

Yes! Testing cortisol metabolism offers a fuller evaluation of the HPA axis in relation to other symptoms and conditions. The metabolism rate of cortisol can be inferred by looking at both free and metabolized cortisol. Cortisol metabolism may be altered in patients with obesity or thyroid conditions and has been reported in other conditions as well. In these conditions, the more complete understanding of cortisol helps improve clinical accuracy. A recent marketing piece from a saliva lab asserts that there is no supporting literature for the fact that cortisol metabolites increase with obesity.

However, Tomlinson6 showed "total glucocorticoid secretion rate was related to both regional and total fat mass and inversely correlated with insulin sensitivity." They went on to show a 36% decrease in metabolized cortisol after weight loss. Kok7 showed that ACTH secretion increases in obesity, which is intuitive if more cortisol is being produced. Our own data, of more than 5,000 women, shows that metabolized cortisol tracks with BMI whereas free cortisol does not (see chart below). Please note that this does not mean that cortisol metabolites have more clinical value than free cortisol. We simply believe that the inclusion of cortisol metabolites gives a more complete picture of the cortisol story for these patients.




A 2013 study showed a reduction in metabolized cortisol of about 30% after weight loss as well. More importantly, they revealed statistical correlation between metabolites of cortisol and waist circumference. His work also showed correlation between cortisol metabolites to subcutaneous adipose tissue and intra-abdominal adipose tissue area (from CT scan at L4). The author also confirmed the correlation between adipose tissue and 11b-HSD type 1 (responsible for activating cortisone to cortisol) activity.8 The data to the right is from an article by Rask that shows a significant increase in the excretion of cortisol metabolites with an increase in body mass index. In 1958, researchers also showed that the metabolic disposal of injected hydrocortisone (cortisol) was faster in obese patients.9

Not only is the absolute number of metabolites relevant, so is the ratio of cortisol and cortisone metabolites (only measurable in urine and included in DUTCH to assess 11b-HSD activity) in the evaluation of weight gain/weight loss and obesity. This metabolic preference for cortisol is also seen with inflammatory conditions and in hypothyroidism.

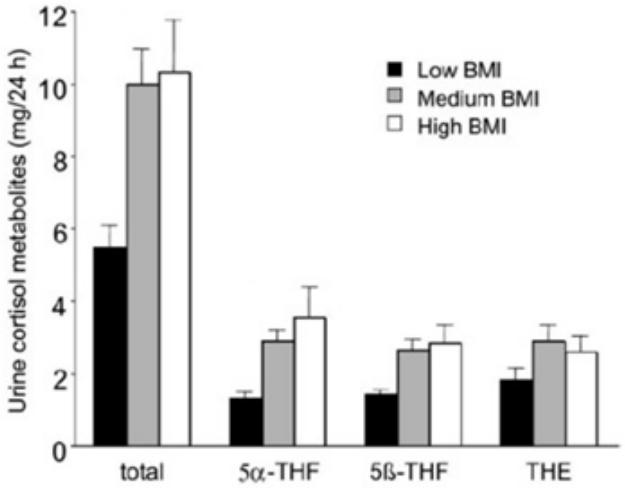
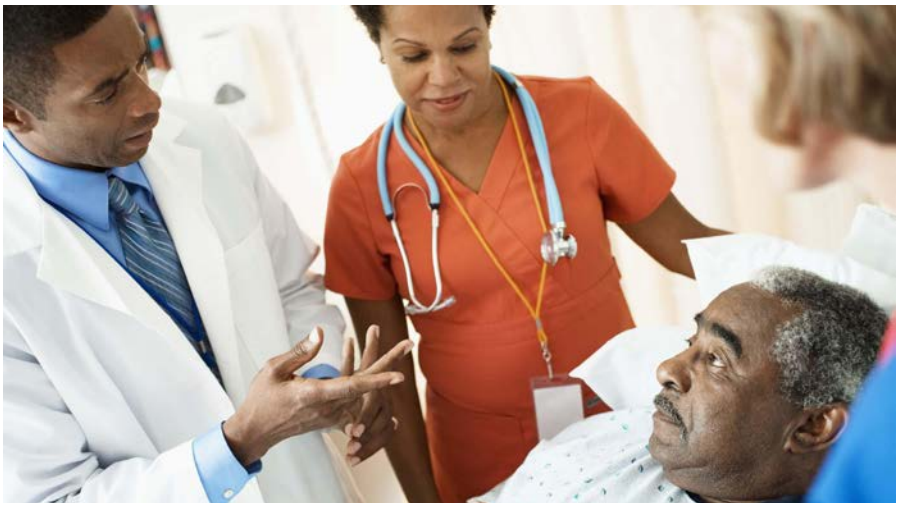



FIG. 3. Urinary cortisol metabolites. Data are the mean ± SE. Subjects were divided into tertiles according to BMI. Total = sum of 5α-THF + 5β-THF + THE + cortols + cortolones. **, Differences between indicated groups, tested with ANOVA and Tukey's B *post hoc* test ($P < 0.01$ for all differences).



## How could abnormal levels of cortisol metabolites (when free cortisol levels differ) impact patients?

When cortisol metabolites are elevated, it implies a high level of cortisol production, even though circulating levels may not be elevated. The reason for this may have to do with obesity, hyperthyroid, an active infection, or some other (possibly unknown) reason. These high levels of cortisol are produced in the outer part of the adrenal gland (cortex) and get pumped through the adrenal medulla at very high concentrations. These concentrations are many times higher than circulating cortisol levels. Is cortisol just passing through the adrenal medulla? No! In this environment, the body makes most of its epinephrine (adrenaline).

Guess which hormone pushes the conversion of norepinephrine to epinephrine? Cortisol! It is an enzyme named PNMT (phenylethanolamine-N-methyl transferase) that is responsible for the conversion, but cortisol stimulates the synthesis of the PNMT catalyst in the adrenal medulla. Here is where this gets both complicated and quite interesting. Wurtman explained, the amount of cortisol pumping through the adrenal medulla (before it has been diluted into general circulation) is at levels 100 times higher than in general circulation.10 The adrenal medulla (first place cortisol goes after production) can have very different cortisol levels than general circulation. For example, if one entirely lacks cortisol production it takes high levels of cortisol therapy (more than 100 times physiological doses) to create physiological PNMT synthesis. At these same doses, peripheral tissues would be flooded with cortisol. In some cases, metabolites of cortisol (which represent overall cortisol production more accurately than free cortisol) may show the amount of medullary cortisol more accurately than free cortisol. High levels of medullary cortisol result in high levels of epinephrine, while long-term elevations may have opposite effects. Though cortisol metabolites themselves may not have significant clinical effects, they allow a window into cortisol production that may not be visible when observing only free cortisol levels.

## Is cortisol metabolism higher in Chronic Fatigue Syndrome?

It is well established that free cortisol is generally lower in patients with Chronic Fatigue Syndrome (CFS). When testing 15 patients with CFS, Jerjes et al. found no difference comparing these patients to 20 controls with respect to their cortisol metabolite measurements.3 One could infer that if free cortisol is lower and total production is not, cortisol clearance is up-regulated. If cortisol metabolites are higher in patients with CFS, this would be a more convincing point. The same author published a larger study the same year. In that study, male patients showed 20% higher levels of cortisol metabolites, and female patients showed 30% higher levels, although neither reached statistical significance. As mentioned before, free cortisol levels were lower. When looking at the ratio between free and metabolized cortisol, female patients with Chronic Fatigue Syndrome showed more than a 70% increase in the ratio of free to metabolized cortisol. In a recent review of HPA-axis function in CFS, the authors state that the cortisol dysfunction related to CFS "may be mediated by...reduced hormone synthesis or increased metabolism."

Low cortisol isn't always present in chronic fatigue. One study mentions it is present in only one third.11 When it is low, increased cortisol metabolism may be part of the picture for some patients. When that patient is your patient, having more information on the overall cortisol picture may be helpful.

## Does thyroid function impact cortisol metabolism?

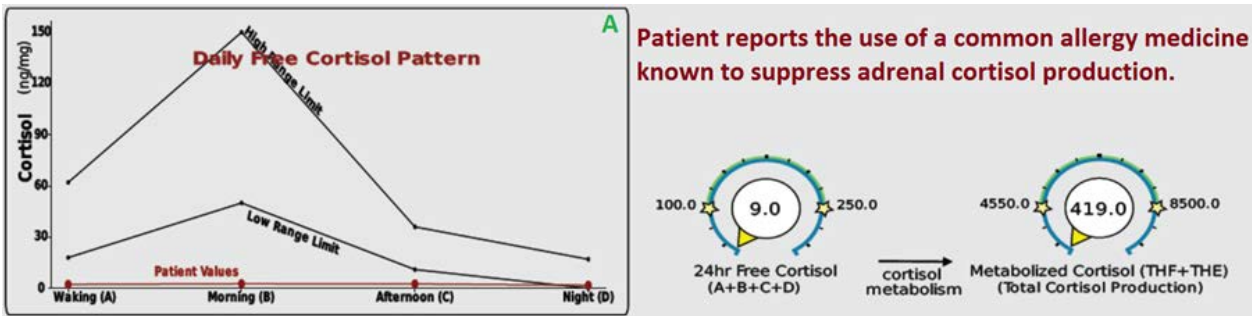
The relationship between metabolized cortisol and thyroid levels is quite profound in the literature. Taniyama12 showed the following data. Graph A shows the relationship with 17-OHCS (a urine test for the total metabolites of cortisol) and thyroid levels (F-T4). Graph B shows the relationship between thyroid status and the cortisol-cortisone balance.

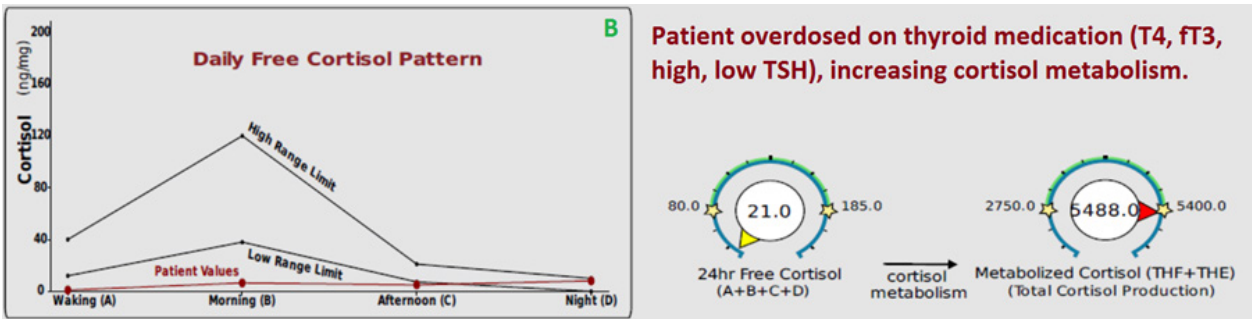
In situations where cortisol clearance may be abnormal (like in obese patients and those with thyroid conditions) a bigger picture is helpful. Consider the following cases where this shows true:

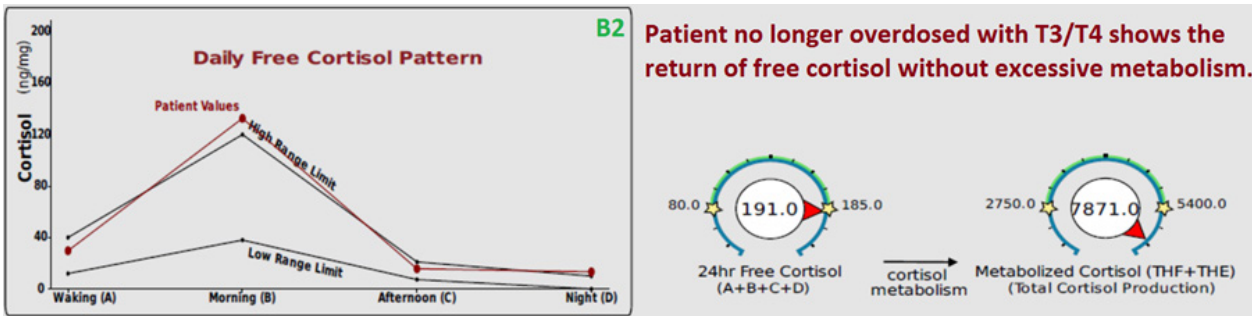
Patients "A" and "B" both show low levels of free cortisol, but for very different reasons. It would be incorrect to assume both have low cortisol production based on consideration of only free cortisol levels. The results for both patients were surprising to the practitioner. Further investigation revealed that Patient "A" had been using Fluticasone, which is a common medication known to suppress adrenal cortisol production. In this case, the metabolites of cortisol confirm low overall production.

Patient “B” also shows low free cortisol, presumably because cortisol is rapidly metabolized due to an overdose of thyroid medication. Serum thyroid labs showed low TSH levels and elevated thyroid hormone levels. This patient was making a lot of cortisol but the excessive metabolism had left her with very low free cortisol. (Patient “A” was male, so reference ranges are different.)

Patient “B” reduced her dose of thyroid medication and free cortisol levels increased dramatically (see Patient “B2”).




## What is the best way to test cortisol for clinical accuracy?

The answer to this question depends on the value one gives to the different pieces of the HPA-axis puzzle that are available. Serum gives you a little, saliva gives you more and DUTCH gives you the most. There may be additional considerations for cost, the ease of patient collection, or other desired information (like sex hormone metabolites) that may be included in the decision making.

If salivary testing includes the CAR (most saliva labs do not offer a true CAR assessment), it has meaningful advantages over urine testing and

may be superior if considering only free cortisol. Urine is a viable alternative for the assessment of the diurnal pattern of free cortisol. We believe the inclusion of cortisol metabolites tips the scale in the favor of DUTCH testing. We find time and again, that practitioners avoid counterproductive clinical intervention by using a more comprehensive option like DUTCH. This is true of cortisol, but also androgens and estrogens as the inclusion of metabolite measurements provides a better clinical understanding. By taking the testing even further with the addition of melatonin, 8-OHdG and other tests, DUTCH provides an unparalleled clinical picture for functional medicine practitioners. With the addition of the CAR evaluation, the DUTCH Plus™ offers even more clinical information for practitioners.

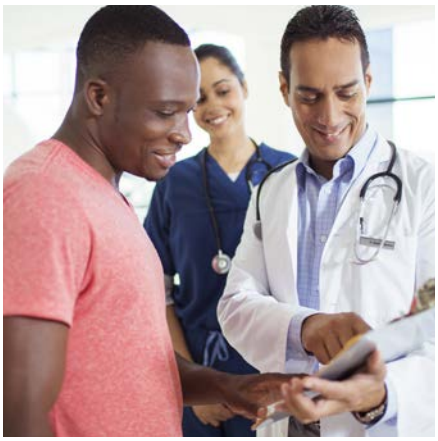
## What is the Cortisol Awakening Response (CAR), and why does it matter?

First, if the CAR is a new concept to you, this is something worth paying attention to, and it is not a simple concept. Over the past few years, the scientific literature has collected an impressive list of studies that show the CAR has independent clinical value in evaluating HPA-axis function. For example, researchers at Northwestern University, showed that while absolute levels of morning cortisol didn't correlate significantly to Major Depressive Disorder (MDD), the CAR did! The difference between the salivary sample taken 40 minutes after waking compared to the waking sample was significantly larger in people who went on to develop MDD.[13]

The act of waking up for the day acts like a mini stress test. While urine and saliva testing can be used to test for the levels of free cortisol at different times throughout the day, the CAR assesses the actual change in cortisol during the first 30 minutes of the day (an additional measurement can be included at 60 minutes). A waking urine sample measures cortisol collected in the bladder during the entire night, while a waking saliva sample offers a cortisol assessment at that moment. Therefore, saliva testing provides a better baseline to assess the Cortisol Awakening Response.

While the CAR is a unique assessment that is only available in saliva testing, few of the saliva labs in the US offer a true CAR assessment. For example, labs like Quest and Mayo Clinic do not offer reference ranges for the assessment of a true CAR. While this offering may be an improvement over salivary cortisol testing of the past, it is somewhat difficult to collect. Continue reading to learn about a new offering from Precision Analytical that includes all the benefits of the DUTCH Complete™, and the addition of the CAR.

## If the CAR assessment has added value and isn't readily available in the market, why not add it to DUTCH testing?



We did! As mentioned above, the CAR assessment cannot be accomplished without salivary samples. We have created a new product we call DUTCH Plus™. This kit includes our typical dried urine collections (without the middle of the night sample), along with five saliva collections, in one simple kit. The DUTCH Plus™ is now available, only through Precision Analytical.

## How does the DUTCH Plus™ compare to other salivary tests?

**Easier collection using swabs (no spitting in tubes)** – It is difficult to collect sufficient volume of saliva in the waking sample using passive drool techniques. Saliva labs are often prohibited from using the more convenient synthetic or cotton swabs because they absorb other hormones that are being tested (like progesterone).

**LC-MS/MS analysis** – a more accurate method than the more popular immunoassays used by most clinical labs. The LC-MS/MS method is less prone to artificially elevated results due to cross-reactivity, includes free cortisone results, and is preferred by labs like the Mayo Clinic.

**The inclusion of free cortisone** – with immunoassays cortisone (the inactive form of cortisol) cannot be included. As with the DUTCH Complete™, the DUTCH Plus™ includes both cortisol and cortisone. This blog post explains the benefits of testing both cortisol and cortisone.

## How does the DUTCH Plus™ compare to the DUTCH Complete™?

The DUTCH Plus™ includes all the markers included in the DUTCH Complete™, but the free cortisol measurements all come from saliva, not urine.

The DUTCH Plus™ includes a 5th free cortisol measurement. Saliva collections at waking, 30 minutes after waking, and 60 minutes after waking allow for the assessment of the Cortisol Awakening Response.

The collection is slightly more burdensome compared to the amazing ease of the DUTCH Complete™ due to the extra samples required. However, the DUTCH Plus™ is put together in a way that is very manageable for patients. In addition, our saliva collection devices are easier to use than traditional devices used by other labs.

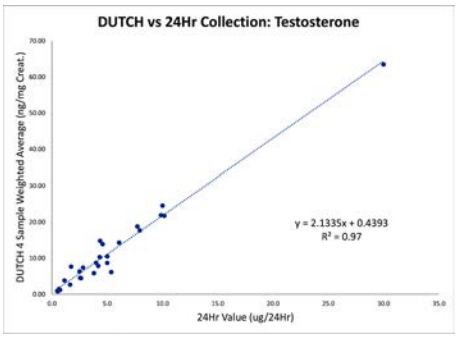
While the DUTCH Plus™ is still very cost-effective, the kit contents, shipping required (express return shipping paid for by Precision Analytical Inc.), and analytical methods make the cost higher.

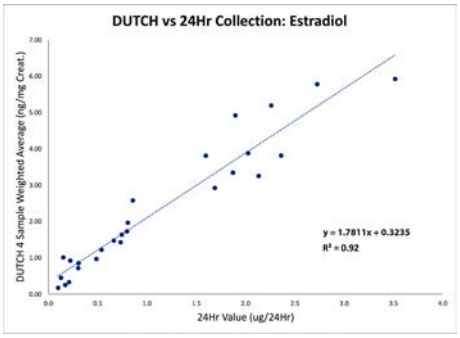
## What is creatinine correction, and does it produce valid results?

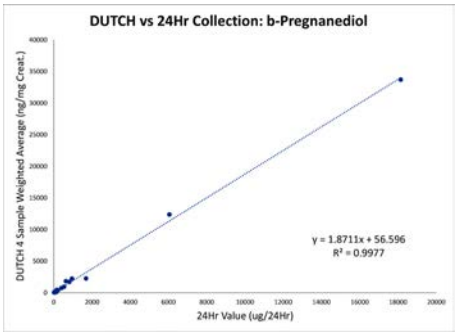
As a urine sample gets more dilute, the concentration of the hormone decreases. The creatinine value decreases in parallel and corrects for hydration. Because creatinine is excreted in a predictable way, this has been used by

labs for many years with respect to hormones. This is not a revolutionary part of what Precision Analytical Inc. is doing. Genova Diagnostics, as an example, reports some of the hormones we test for -- along with over 150 other markers in liquid urine -- all using creatinine to correct for hydration.

We work with the assumption that a 24-hour collection is the gold standard for urine hormone testing, except for melatonin (a nocturnal production of melatonin may be a better measurement than a full 24-hour collection). A 24-hour collection can be difficult to complete correctly (or at all if the patient isn't up for the challenge). If a 24-hour collection is done correctly, DUTCH values compare favorably. This correlation data is critical in concluding that creatinine correction, as used in the DUTCH test, is effective for producing results reasonably equivalent to 24-hour urine values.



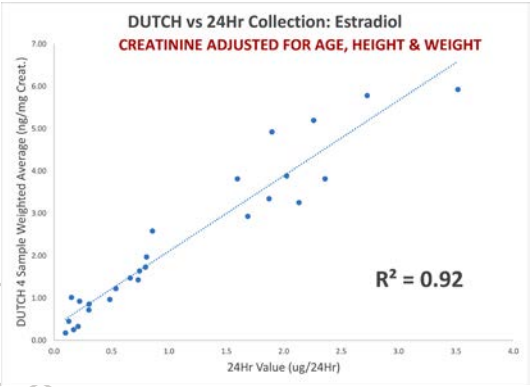



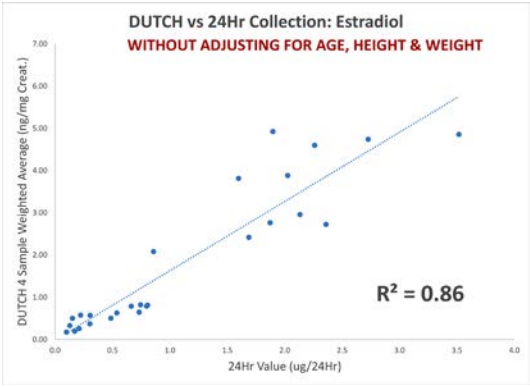




## If creatinine changes with age, height and weight, does this introduce bias into spot-urine testing?

It is true that creatinine is dependent on the age, height and weight of the patient. Because the relationship between creatinine excretion, age, height, and weight have been well defined, we can correct for these variables.

One can see above how the correlation to a true 24-hour collection is improved by correcting for this bias. This is not something known to be done by other labs using a spot urine model, but it improves the clinical accuracy of the testing. When looking at an entire population of data, this adjustment makes a small overall difference, but the difference in uniquely large or small individuals can be significant.



## If the patient has abnormal creatinine clearance, can it invalidate the results?

If a patient has abnormal creatinine clearance, it could affect the results. 24-hour urine tests are dependent on people collecting their samples correctly and measuring the total volume accurately. A study by Tanaka showed as many as 40% of patients did so in error14. The DUTCH test doesn't require the same level of patient compliance. However, there may be uncertainty regarding creatinine in some cases. If the patient has a known kidney (or other) condition that may impact creatinine clearance, urine testing may not be the best option (or results should be interpreted carefully). If creatinine excretion is "off" it will impact the results. This should be considered if DUTCH results seem to show an elevation or deficiency across all the numbers that may not make sense clinically. This would be analogous to the patient that collects a 24-hour urine test incorrectly, missing 20% of the urine for the day or incorrectly measuring the total volume. It is also noteworthy that any urine test (DUTCH or 24-hour) should not be used for free cortisol assessment if the patient is known to have an abnormal glomerular filtration rate (cortisol may be artificially low in cases with low GFR).

## Is measuring the primary melatonin metabolite (6-hydroxymelatoninsulfate) in a morning urine sample a valid means of evaluating melatonin production?

Let's just go right to the literature for this one. Schernhammer and Hankinson concluded, "The urine concentration of the major metabolite of melatonin, [6-hydroxymelatoninsulfate], is highly correlated with melatonin levels in blood and saliva. Urinary levels, as measured in first morning urine, accurately reflect plasma melatonin levels measured during the previous night." The authors continued by concluding "our findings indicate that melatonin secretion, as assessed by [6-hydroxymelatoninsulfate] levels in first morning urine, may play an important role in breast cancer development." Their study showed that patients with higher levels of nocturnal melatonin measurement were less at risk for breast cancer.15

Urine measurements have an advantage over saliva testing in that melatonin levels may peak for one patient at 1am and for another at 3am. Urine testing correlates with the total for the entire night and doesn't require waking in the middle-of-the-night for collection. Precision Analytical is not unique in this measurement. Other clinical labs (most of which offer blood and saliva testing as well) find this urine marker as useful as we do. Urine testing of 6-hydroxymelatoninsulfate does not reflect systemic levels of melatonin when supplementation is given due to extensive first-pass metabolism. In these cases, the urine values are very high and not clinically relevant. As with most lab tests, urinary testing of 6-hydroxymelatoninsulfate has its pros and cons, but it clearly has added clinical value overall and is a valuable addition to DUTCH.

## Is the DUTCH test FDA-approved?

FDA-clearance (510(k) clearance) is typically for the development and sale of laboratory test kits that are manufactured by one company and then sold to clinical labs to use in their testing. For example, a blood or saliva cortisol test is usually

performed by an immunoassay kit. These immunoassay kits are made by a handful of manufacturers and sold to hundreds of labs (such as our competitors) to run the test. These tests cannot be sold to clinical labs unless they are FDA-cleared. The other option used is liquid chromatography tandem mass spectrometry (LC-MS/MS). LC-MS/MS methods are considered a laboratory-developed test (LDT) because they must be developed "in-house" by each lab that uses them. These types of LC-MS/MS tests are not governed by the FDA and must be validated by the testing lab. Because of the advantages of using LC-MS/MS (increased accuracy and the ability to test free cortisone along with cortisol), labs like Mayo Clinic and Quest Diagnostics choose to use LC-MS/MS as their method of choice for salivary cortisol. For their purposes, the advantages of LC-MS/MS outweigh the benefits (FDA-cleared, less expensive, easier to scale) of using an immunoassay.

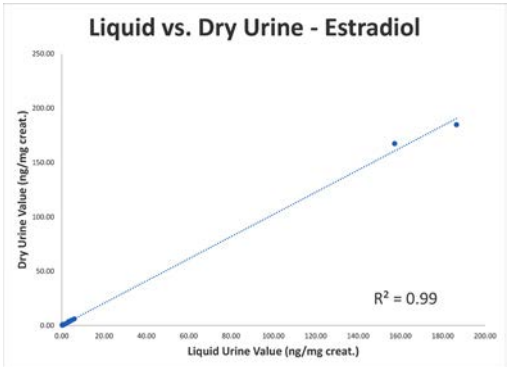
Since we have chosen mass spectrometry for each of the DUTCH tests they fall under the usual oversight of any LDT test. The FDA does not govern these types of tests. Clinical Laboratory Improvement Amendments (CLIA) reviews all our DUTCH tests, their validation data, and our QA/QC programs in their regular inspections. This is the norm for labs performing urine testing of steroid hormones and their metabolites.

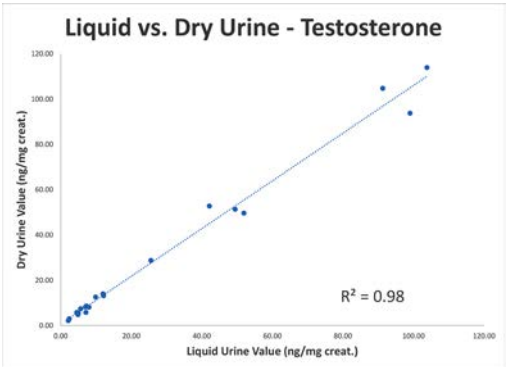
## Is the filter paper used for sample collection FDA-approved?

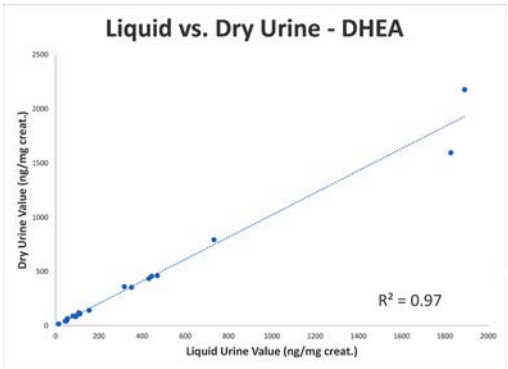
The filter paper used in the DUTCH collection is FDA-approved to collect whole blood samples. It is FDA-approved for the collection itself and not specific tests. The FDA-approval status is important because it insures the quality and consistency of the manufacturing. You will find many laboratory-developed tests in the market where bloodspot collections are used for various tests. For example, years ago, our founder Mark Newman developed the world's first bloodspot vitamin D assay (25-OH-vitamin D2 & D3) at ZRT Laboratory. The filter paper is approved for the collection of whole blood, but the entire assay had to be validated (accuracy, precision, serum correlation, etc.). You can see the initial method published with some of our data.16 The initial method was later vastly improved upon.17 The filter paper worked all along, but the quality of the data depended on the quality of our analytical method. The filter paper has been shown to hold a certain volume of blood per area of filter paper, which is important for blood testing. Urine testing is much easier and more forgiving because slight differences in the amount of volume held by the paper are corrected by reporting relative to creatinine (more on this in the next section).

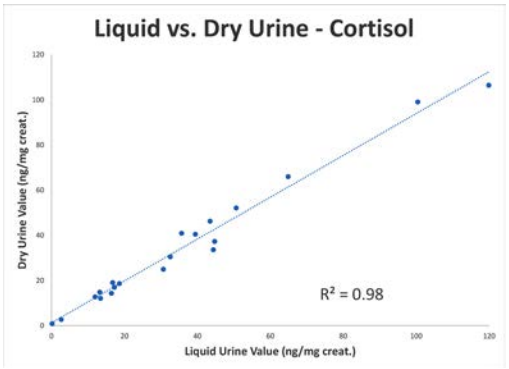
While there has been no formal FDA approval process for using this filter paper for urine samples to date, a cohort of labs has validated over 100 different tests using this filter paper. There are simply not enough labs using this filter paper for urine collection to justify the expense for the companies selling the paper to acquire FDA-clearance for dried urine (as opposed to whole blood), but it will likely be a future goal. Because we are not manufacturing the product, it is not within our discretion to apply for clearance to use the paper for urine collection. As such, it would be inappropriate to use the paper for urine collection without a thorough validation to ensure that accuracy and precision are maintained when using dried samples. This validation has included various studies that have been completed for each hormone

tested. CLIA prefers we put a statement in our comments letting people know that because they are intended for blood it is technically considered “research only” and must be validated. Validating the accuracy of dried collections, relative to liquid urine, includes comparison and stability studies. The graph below is an example of recent data collected comparing dried and liquid urine samples that were tested for cortisol. Similar plots for each hormone show excellent correlation and accurate testing with dried samples. As someone who has worked with dried samples for almost 15 years, we have found some compounds that do NOT work as dried samples. For example, some compounds degrade during the drying process if they have a functional group that is unstable. Even if the paper is FDA-approved for urine collection, each test must be independently validated to ensure accuracy. In the graphs below you can see representative data for the equivalence between liquid and dried analysis of urine hormones and their metabolites.



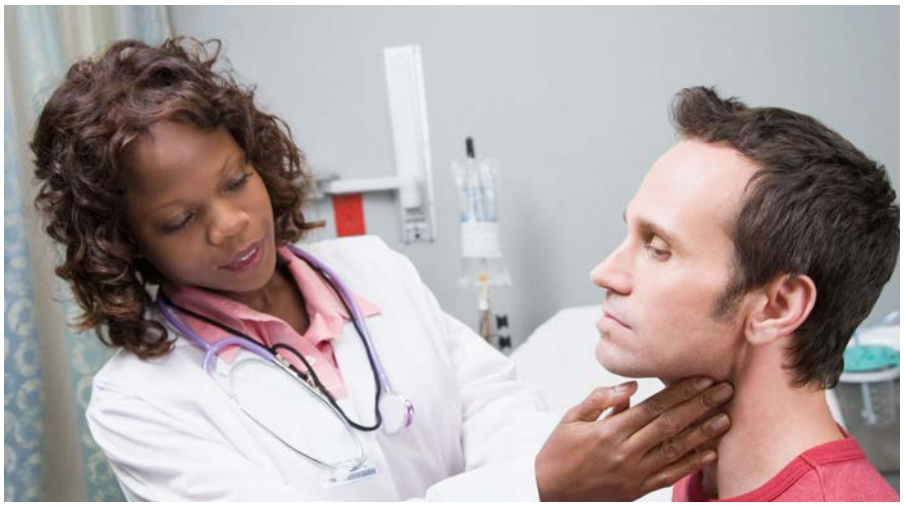




Most labs using dried samples face the challenge of having minimal sample to work with (~2mL on each collection device.) To combat this, Precision Analytical Inc. uses the most sensitive and sophisticated analytical methods available. One objection to our method claims, “there is no way to get all of those values with such a small sample.” However, even with a small sample, we can see low-level hormones better than many of the traditional methods still used by labs testing liquid urine. There is simply no need for additional sample for any of the hormones measured, due to the sensitivity of the DUTCH test.

CLINICAL VALIDATION OF DUTCH TEST

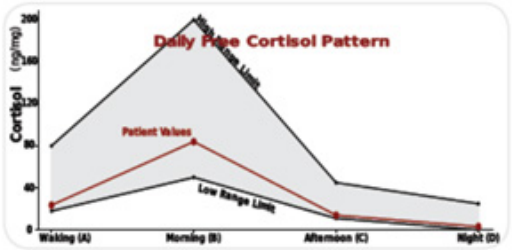


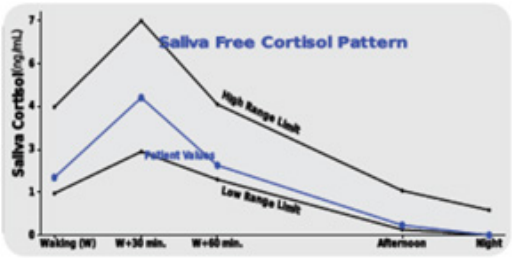
## APPENDIX

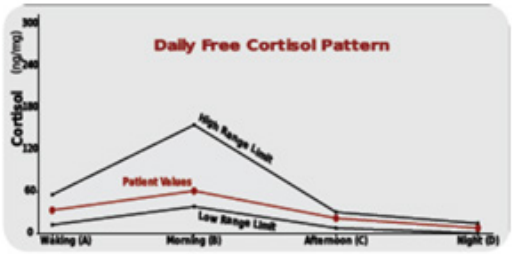
### Urine and saliva parallel data for cortisol measurements:

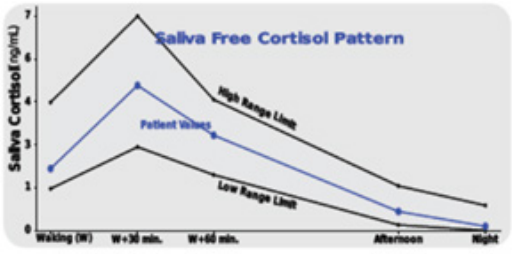
The following show parallel patterns for individuals collecting saliva and dried urine samples on the same day. Remember, the first data point for urine testing has no saliva parallel. This “A” sample in urine represents the sleeping period. The next urine sample (“B”) represents the entirety of the first two hours of the day. The first three saliva samples are collected in this period and in aggregate they represent a similar period as the second urine measurement.

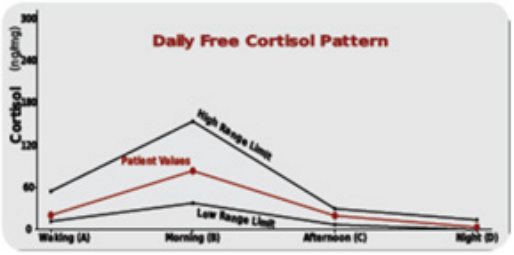
**“Normal” cortisol patterns:** Here are six urine and saliva matches from individual with relatively “normal” patterns throughout the day. These patients not only have “normal” values, but the magnitude of the CAR is also an expected value. In these cases, the CAR adds confirmatory value but not additional value to the testing.

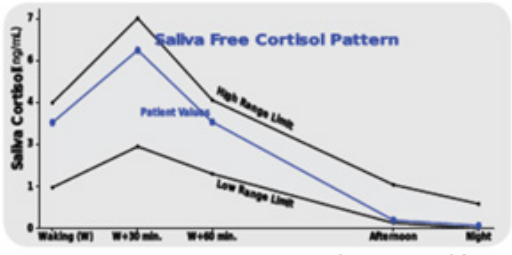


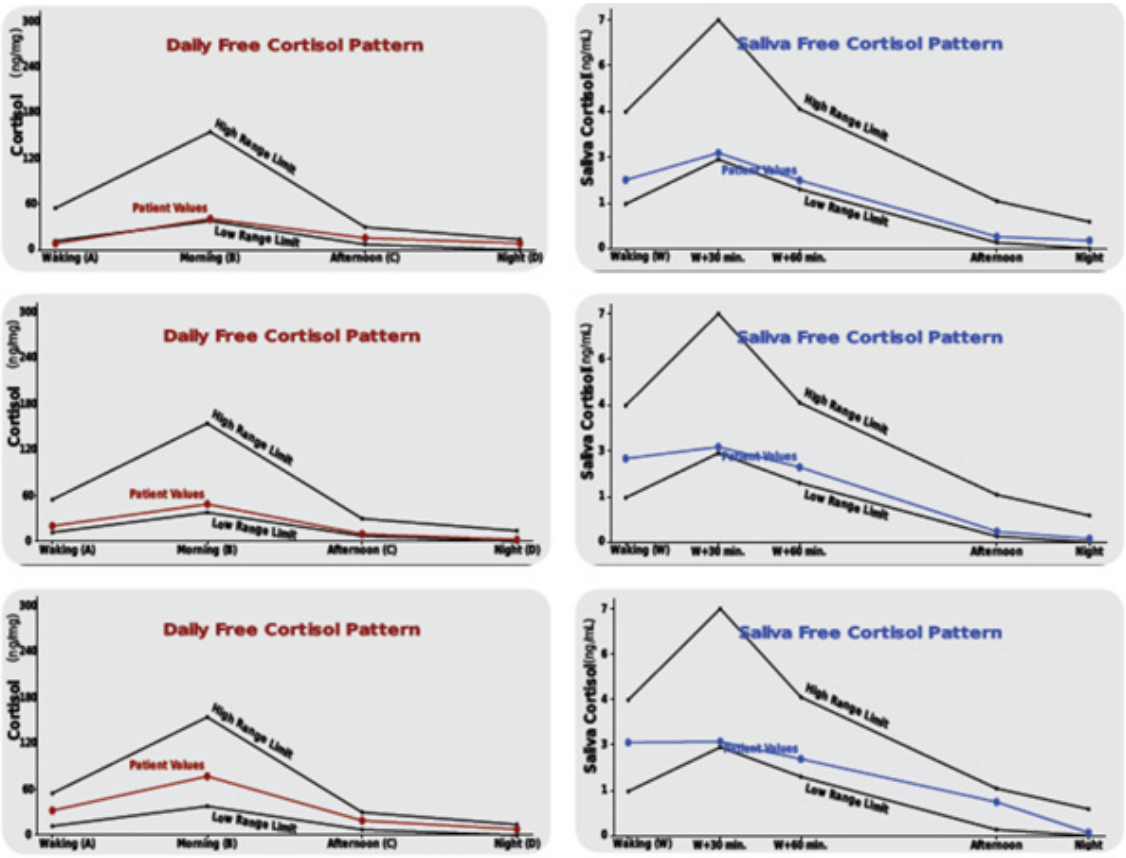



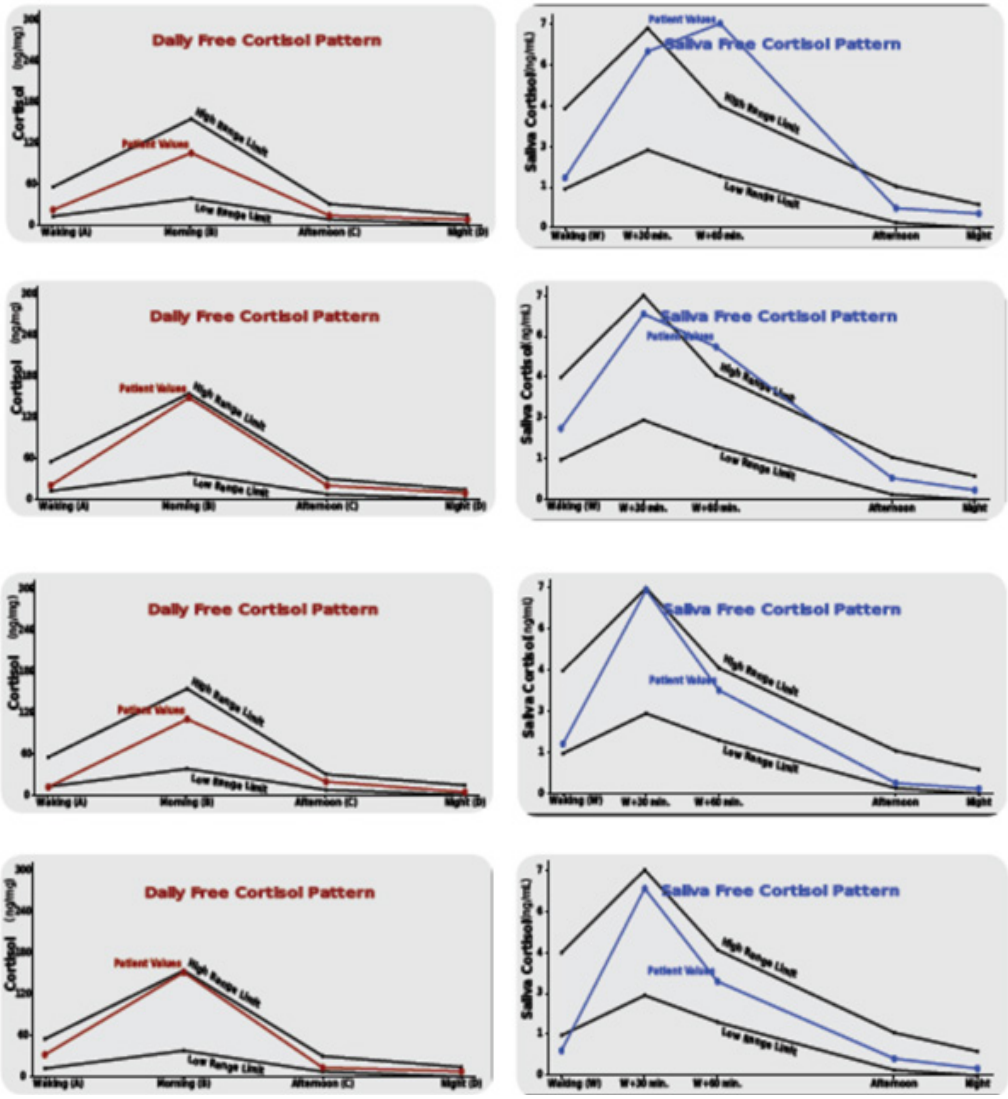



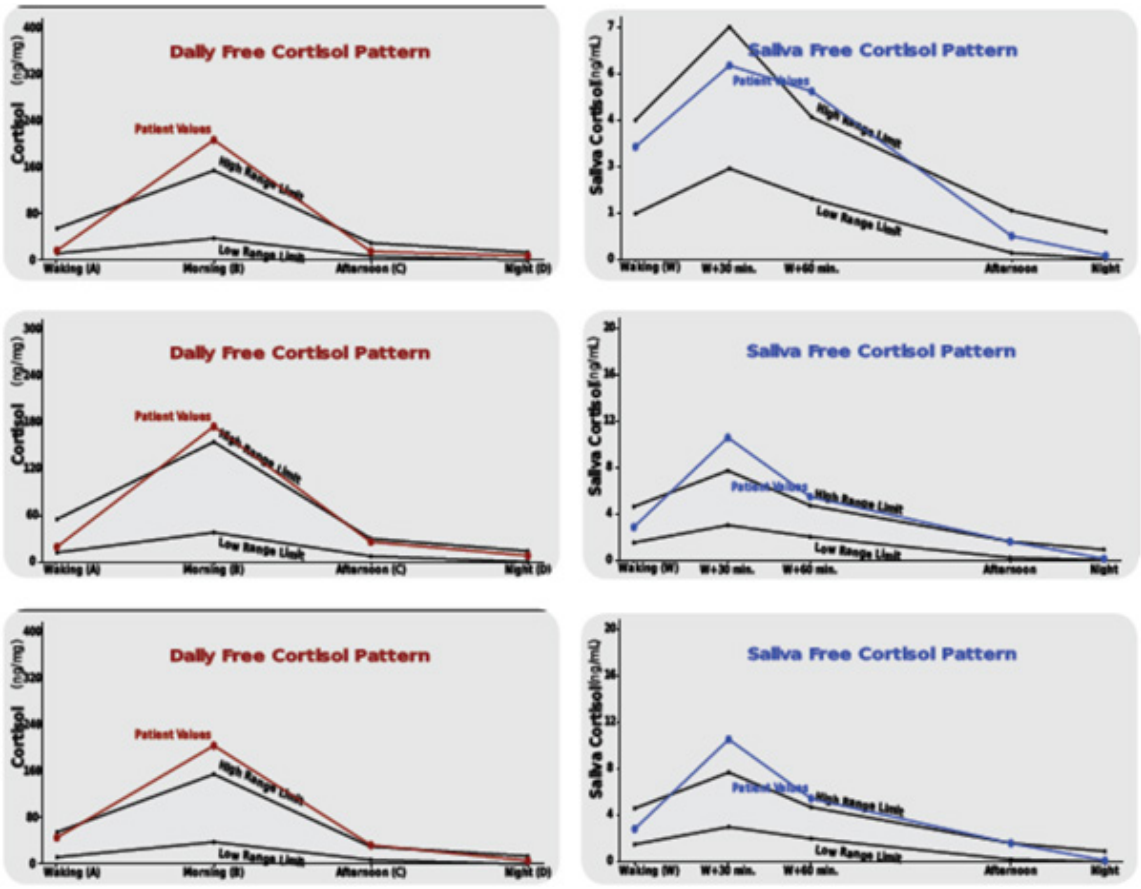
**Call me "normal" but don't call me a morning person!** These patients show some added value for the CAR. Their overall up-and-down patterns are "normal" but the actual response to waking is blunted. This may imply an HPA axis that doesn't respond as it should when faced with a stressor. If the patient has symptoms of low cortisol or a poor stress response, you may have uncovered something with the CAR that would have gone hidden with traditional saliva testing or the DUTCH Complete.



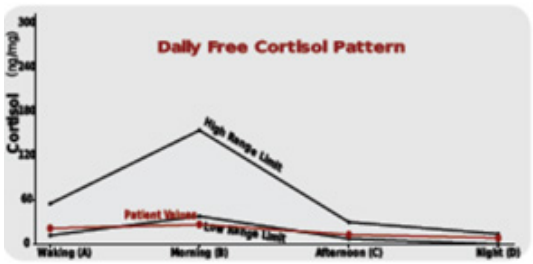
**You may not know it, but I'm a little stressed:** These patients show normal diurnal patterns, but they show an exaggerated CAR. None of the results are elevated at waking or 30 minutes later, but the magnitude of the jump in the morning is bigger than expected (and some show elevations 60 minutes after waking). This is exactly what Adam, et al. found to be predictive of developing MDD (major depressive disorder).13 The larger than expected CAR is thought to be from a patient with a high level of perceived stress or an HPA axis that overreacts to a stress event. In these cases, the CAR adds to the clinical picture in a way that will be helpful in some patients.

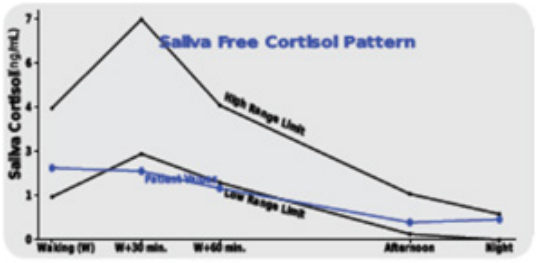


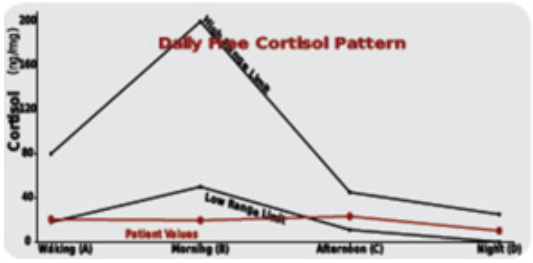
**No need for morning coffee:** This is a profile, I often see in practitioners with busy, stressful practices. Their overall patterns are reasonable but the early morning samples show high levels of cortisol. The CAR shows similar patterns. In one example, you can see that the sample collected 60 minutes after waking was even higher than the +30-minute sample. This points to either a very high level of perceived stress or a very overactive HPA axis. As is common in what I've seen, this sample came from a practitioner testing herself. The results spoke very clearly to her clinical picture and is common in high performing successful (but stressed!) professionals. These types of patterns will predispose patients to the development of clinical manifestations of the high cortisol results.

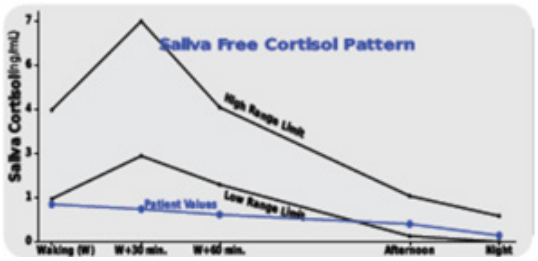


**Pass the coffee in the morning:** Whether looking in urine or saliva, you can see a profound lack of cortisol in the early morning. Does the CAR help us here? It does. You can see in the first three examples there are low overall levels and essentially no discernable CAR. These patients are in tough shape. The other four have low overall levels, but you can see some HPA axis activity, albeit somewhat subdued. The distinction between these two subgroups may be significant in some cases in terms of prioritizing treatment and the aggressiveness with which treatments are pursued. It may also alter the relative consideration of using hydrocortisone treatment.

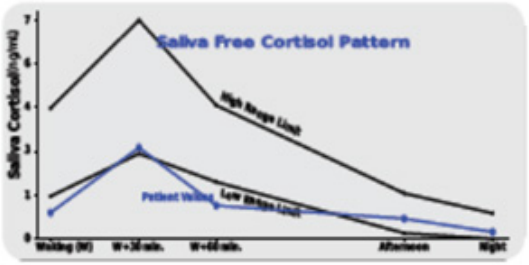


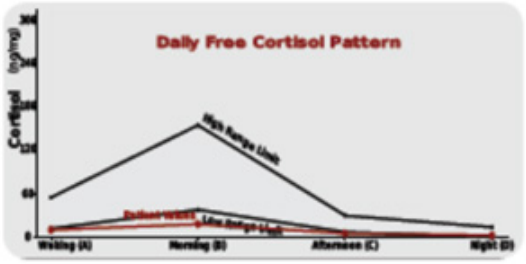



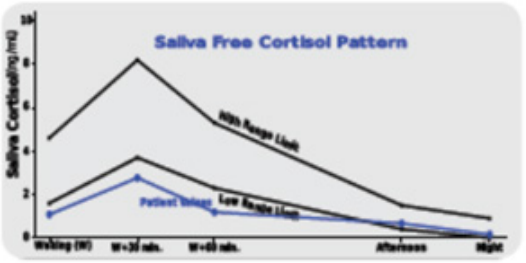



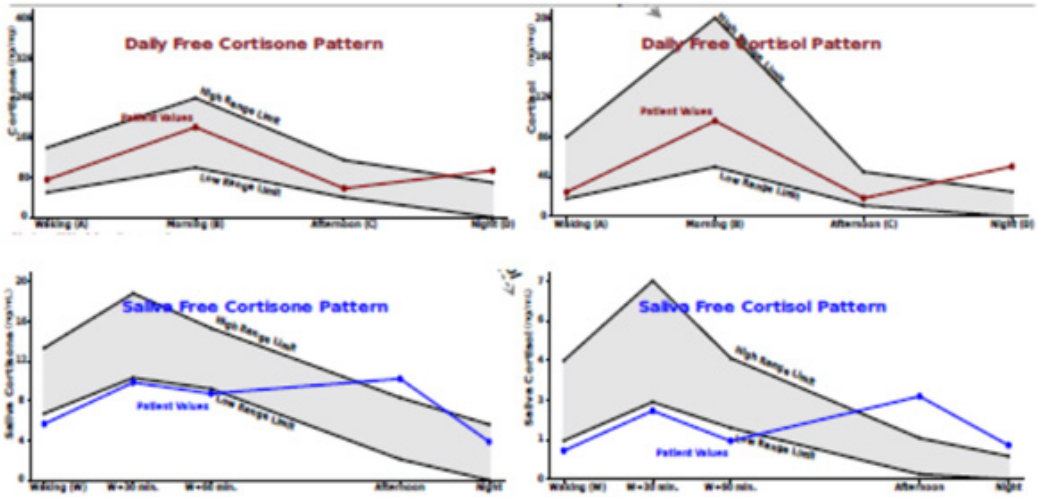




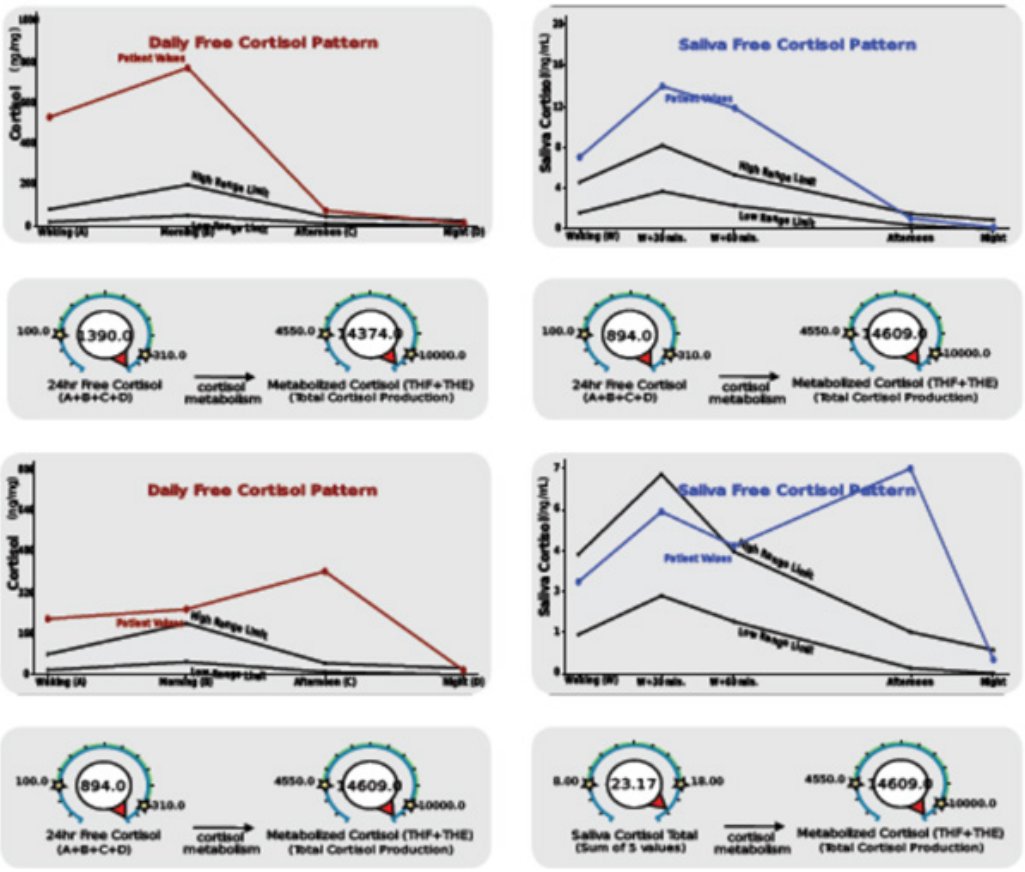



**Good luck getting to sleep:** These patients show normal levels (two are normal, one is a cortisol mess) during the daytime but have a cortisol result that kicks up at bedtime. For these patients, it is important to look at cortisone as well as cortisol. Why? What if the patient used hydrocortisone cream (which IS cortisol) at bedtime and contaminated the samples? The elevated cortisol results (for both urine and saliva) are legitimized by the concurrently elevated cortisone. If the samples were contaminated (which we see from time to time) the cortisol would increase but cortisone would not (a great reason to use an LC-MS/MS assay that includes cortisone).



**Call the fire department:** These patients are on fire with cortisol. Notice that they both come down at bedtime, so it's not Cushing's Disease. Urine results show that both make a lot of cortisol even while sleeping (high waking urine free cortisol). One of the patients shows higher levels in the afternoon. The other patient shows extremely high morning levels. This patient went on to develop (for the first time) depression, which makes sense with all this cortisol. This patient's health issues were more recent and quite profound. The other patient suffered from longer term conditions (multiple sclerosis among others). Does the CAR help in these cases? Both patients show a functional CAR, but it is just turned up on high. The CAR data likely doesn't change the clinical plan or outcome in these two cases. Similarly, I included the cortisol metabolites for these two. No big surprise, they are also high. In many cases the metabolites add to the clinical picture. Like the CAR, when there is a profound deficiency or elevation across the board for cortisol, just about any test will tell the right story. For many cases where cortisol dysfunction in more nuanced that the DUTCH additional information (metabolites or CAR) helps to clarify.



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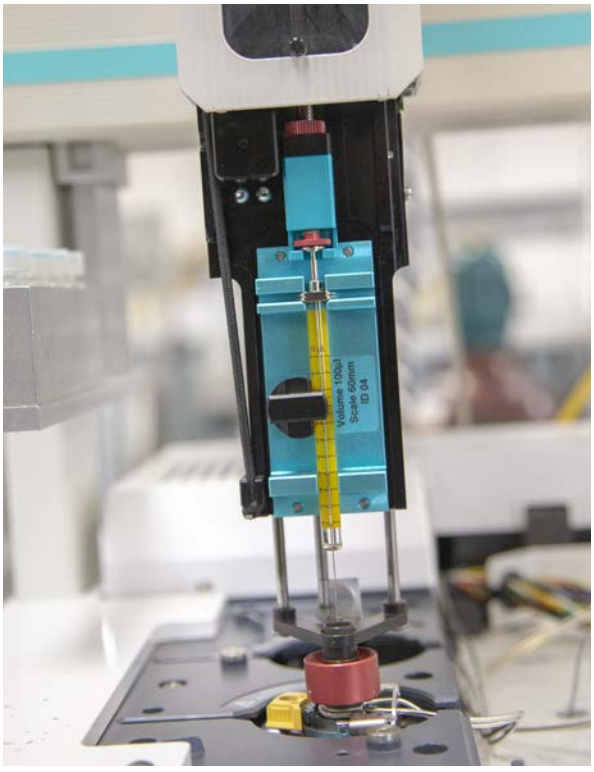
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