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High resolution daily profiles of tissue adrenal steroids by portable automated collection

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Overline: CIRCADIAN RHYTHM

One sentence summary: This study represents a mathematical analysis of the normal variability of 24-hour adrenal steroid hormone profiles in ambulatory healthy adults, quantified using a wearable microdialysis device and high-performance liquid chromatography-mass spectrometry.

Abstract:

Rhythms are intrinsic to endocrine systems, and disruption of these hormone oscillations occurs at very early stages of disease. Because adrenal hormones are secreted with both circadian and ultradian periods, conventional single time point measures provide limited information about

rhythmicity, and crucially do not provide information during sleep when many hormones fluctuate from nadir to peak concentrations. If blood sampling is attempted overnight, this necessitates admission to a clinical research unit, can be stressful, and disturbs sleep. To overcome this problem and to measure free hormones within their target tissues, we used microdialysis, an ambulatory fraction collector, and liquid-chromatography tandem mass-spectrometry (LC-MS/MS) to obtain high-resolution profiles of tissue adrenal steroids over 24 hours in 214 healthy volunteers. For validation, we compared tissue against plasma measurements in a further seven healthy volunteers. Sample collection from subcutaneous tissue was safe, well tolerated, and allowed most normal activities to continue. In addition to cortisol, we identified daily and ultradian variation in free cortisone, corticosterone (CCS), 18-hydroxycortisol (18-OHF), aldosterone, tetrahydrocortisol (THF), allotetrahydrocortisol (aTHF), and the presence of dehydroepiandrosterone sulphate (DHEA-S). We used mathematical and computational methods to quantify the interindividual variability of hormones at different times of the day and develop "dynamic markers" of normality in healthy individuals stratified by sex, age, and body mass index (BMI). Our results provide insight into the dynamics of adrenal steroids in tissue in real world settings and may serve as a normative reference for biomarkers of endocrine disorders (ULTRADIAN, NCT02934399).

INTRODUCTION

The hypothalamic-pituitary-adrenal axis (HPA) is a neurohormonal system fundamental for life. Not only does it play a vital role in the regulation of cognitive, metabolic, and immune function, the HPA axis is also a critical part of the body's response to stress (*1*, *2*). Cortisol –the principal glucocorticoid in humans – is secreted from the adrenal cortex dynamically in response to pulsatile release of pituitary adrenocorticotropic releasing hormone (ACTH) (*3–5*). In addition to cortisol, the action of ACTH on the adrenal glands results in the production of multiple hormones and their precursors including corticosteroids, androgens, and mineralocorticoids (*6–8*). This produces a complex and dynamic adrenal hormone milieu which varies greatly across different times of day. High frequency blood sampling demonstrates ultradian cortisol secretion in humans (*9, 10*), with a pulse frequency of 60-90 minutes (*11, 12*). Ultradian pulsatility occurs within a circadian-modulated framework in which cortisol rises in anticipation of awakening, peaks shortly afterwards as daily activities begin, then falls to a nadir at the end of the day, usually corresponding to the onset of sleep (*13*). The overarching daily rhythm of glucocorticoids is coordinated by a central pacemaker located in the suprachiasmatic nucleus of the hypothalamus. Mathematical modelling predicts (*14*) and *in vivo* studies confirm (*4*) that ultradian pulsatility is the result of systems-level feedback interaction between corticotroph cells in the anterior pituitary and steroidogenic cells in the adrenal zona fasciculata.

Like cortisol, aldosterone is also released in a pulsatile manner, and through its actions on the mineralocorticoid receptor acts to regulate sodium haemostasis and blood volume. The generally

held belief is that aldosterone secretion is primarily determined by the renin-angiotensin system, at least in normotensive states (*15*), although plasma aldosterone correlates better with plasma cortisol than with plasma renin activity (*16*). Indeed, studies of aldosterone support a much more complex picture where aldosterone may also be influenced by sleep (*17*, *18*) in addition to modulation by ACTH (*6*), potassium intake (*19*), and renin (*20*).

Disruption of normal rhythmic adrenal steroid secretion is associated with wide ranging effects on metabolism, behaviour, and cognition (*13*), as well as endocrine diseases such as Cushing's (*21*) and primary aldosteronism (*22*). However, inter-individual and time-based variation in hormone concentrations across the day results in population-based reference ranges that are very wide. Consequently, it can be difficult to make diagnostic decisions based on single time point samples. In addition, single time point measurements fail to capture important hormone changes that occur during sleep - typically the time of day of adrenal hormone nadirs, least variance, and when the fasting state can be observed without interference related to physical activity. Likewise, although detailed examination of urine collections provides insights into overall changes in steroid production and metabolism (*23*), urine is necessarily an integrated measure and cannot provide fine-grained detail about dynamic features, especially if the collection sample covers a full day.

To make substantial progress in understanding the importance of dynamics in human endocrinology, information needs to be gathered at high frequency and with minimum disruption. Dynamic profiling using multiple blood samples provides significantly more information than single samples (*24*) but is not practical as a routine diagnostic or monitoring tool due to its invasiveness, non-naturalistic setting, heavy resource requirement, potential sleep disruption and the inconvenience to the patient.

Our approach was to develop a patient-acceptable, portable sampling system (*25–27*) that delivers automated dynamic hormone profiling with high resolution and minimal inconvenience. In addition, we aimed to provide a system with the flexibility for use as an outpatient, including in the home, allowing sampling in more naturalistic settings. Microdialysis was an attractive solution in that measurements may be obtained at a similar temporal resolution to inpatient blood sampling, but without the need for blood. A further advantage of microdialysis is that it measures tissue concentrations of the free, and thus biologically active fraction of the relevant hormone, in contrast to blood samples that measure total (protein bound + free) concentrations of hormones (*28*).

We developed a fraction collector, which when combined with ultrasensitive liquid chromatography tandem mass spectrometry (*29*) and mathematical methods, allowed us to demonstrate the viability of ambulatory microdialysis to quantify the healthy daily variability of tissue-free hormones in interstitial fluid. We validated this *corticosteroidome* against

simultaneously collected samples in plasma and provide the normal ranges needed for the foundation of a system of dynamic endocrine diagnostics.

RESULTS

U-RHYTHM ambulatory microdialysis reveals 24-hour dynamic profiles of multiple adrenal steroids

Using the U-RHYTHM ambulatory microdialysis sampling system (Fig. 1A-B) we obtained daily profiles of adrenal steroids in 214 ambulatory healthy volunteers. Samples were collected every 20 minutes providing 72 samples (24 hours) of data per participant. In each sample, multiple adrenal steroids were identified across a wide range of concentrations. From these, we constructed dynamic profiles for tissue cortisol, cortisone, 18-hydroxycortisol (18-OHF), aldosterone, corticosterone (CCS), cortisol metabolites allo-tetrahydrocortisol (aTHF) and tetrahydrocortisol (THF), and dehydroepiandrosterone sulfate (DHEA-S) (Fig. 1 and Fig. S1). Cortisol, cortisone, and 18-OHF profiles were detected in 100% of participants and in >97% of the total sample population overall. Aldosterone was detected in 81.4% of all samples forming profiles in 88% (188/214) of participants. CCS was present in 76% of samples forming profiles in 81% (173/214). Hormones measurable in <50% of samples included tetrahydrocortisone (THE) (20.1%) and 11-dexoxycortisol (11DOC) (18.0%). The most abundant steroid was DHEA-S and the least abundant aldosterone (Table 1, Fig. S2). Hormones detectable in only a few samples included allo-tetrahydrocortisone (aTHE) (1.6%), 21-dexoxycortisol (21DOC) (0.69%), and 18-oxocortisol (18oxoF) (0.60%). These hormones were not considered for further analysis (Table S1).

Subcutaneous adrenal steroids exhibit circadian and ultradian rhythms

Inspection of the hormone profiles suggested that there was a daily rhythm of all steroids, except for DHEA-S (Fig. 1 and Fig. S2), with nadir concentrations occurring after onset of sleep and peaks around wake time. Given that this pattern is consistent with the daily response of the adrenals to ACTH (*1*), we formally quantified rhythmicity of the observed adrenal steroid profiles using non-linear cosinor regression analysis. We examined 1,498 24-hour hormone profiles (7 hormones x 214 participants) using sinusoidal functions and estimated the best population-level fits for each hormone (*30*). This cosinor analysis (Fig. 2A-G) provided very strong evidence of a daily rhythm of tissue cortisol, cortisone, 18-OHF, corticosterone, aldosterone, aTHF and THF ($p<0.001$), but not for DHEA-S. In addition, we found evidence to support ultradian rhythmicity. The cosinor method determined that a multiple component model including an 8-hour period ($p <0.001$, Fig. 2A-G and Table S2) was the best fit for the rhythmic hormone profiles.

We compared the similarity between dynamic hormone profiles by means of stationary pair-wise cross-correlation of median hormone concentrations at each time point, across the 24-hour time series (Fig. 2H and Figs. S3-S10). We used the median because data were not normally distributed across most time points. For reference, the correlation of the means is shown in Fig. S3. Using tissue cortisol as a reference, we observed highly positive cross-correlations between all rhythmic hormones (r_s = 0.77-0.99, p<0.001). The strongest correlations were between the median concentrations of cortisol and 18-OHF, cortisol and cortisone, cortisol and CCS, and 18-OHF and CCS (all r_s >0.9). Median concentrations of aldosterone were also highly correlated with both cortisol and 18-OHF (r_s = 0.93 and 0.91 respectively). Metabolites of cortisol (aTHF and THF) were also highly correlated with each other (r_s = 0.99), and with cortisol (r_s = 0.86 and 0.89 respectively).

Having identified both cross correlation and rhythmicity of tissue corticosteroids across the day, we calculated the distribution of hormone peaks and nadirs in relation to clock time and daily events, including sleep and mealtimes (Fig. 3A, Table 2). Following their nadir, hormone concentrations began to rise several hours prior to waking. The population peak of cortisol, cortisone, CCS and 18-OHF occurred within the first 60 minutes of waking, whereas aldosterone reached peak concentrations approximately 2 hours after wake time (Fig. S11). Concentrations of hormones exhibited a gradual decline across the day, except for aldosterone, where mean concentrations remained higher between waking and 6pm compared with the other steroid hormones (Fig. 3A and Figs. S12-S18). We found that for all rhythmic hormones, concentrations fell to nadir after the onset of sleep, rather than before (Table 2).

Correlation of cortisol with aldosterone exhibits high interindividual variation

Concentrations of tissue aldosterone were overall low, becoming undetectable at night in some individuals (Fig. S1, Table S1), and profiles were characterised by high interindividual variability (Fig. 1). Given the strong correlation of the population median concentrations of aldosterone with cortisol across the day, we investigated this interindividual variability in more detail by computing the Spearman correlation of the two hormones for every available ambulatory profile (n=188). We found that in many profiles aldosterone was highly correlated with cortisol across the entire 24-hour period (example in Fig. 3B) but in other cases the profile of aldosterone was more distinctly pulsatile, resulting in weak or absent statistical correlation with cortisol (example in Fig. 3C). Overall, the median individual correlation of aldosterone with cortisol was 0.59 (25^{th} – 75^{th} centiles 0.41 – 0.75, Fig. S19A). By contrast, examining the relationship of 18-OHF with cortisol in the same way (n=214) revealed consistent high correlation (median r_s = 0.90, 25^{th} – 75^{th} centiles 0.84 – 0.94, Fig. S19B).

Tissue 18-hydroxycortisol is phase advanced with respect to cortisol and cortisone

Having established the distribution of peak and nadir times for rhythmic hormones at a population level (Table 2) we examined time varying relationships of specific hormones in more detail. We computed the time lagged cross correlation between cortisol and cortisone, and between cortisol and 18-OHF, for each individual profile. Cortisol and cortisone were highly synchronised, lagging cortisol by a mean of 8.2 ± 18 minutes. In contrast, cortisol lagged 18-OHF by a mean 35.3 ± 24.9 minutes (Figs. S20A and S20B).

Dynamic hormone profiles in ambulatory vs non-ambulatory participants

We compared the ambulatory results with profiles of hormones measured simultaneously in plasma and tissue under laboratory conditions in which participants remained predominantly semi-recumbent and inactive (n=7, Fig. 4 and Figs. S21-S28). All hormones detectable in the ambulatory cohort were also recovered, although in the microdialysate aldosterone and CCS were below the limit of quantification in multiple samples. Measurements of 18-OHF and CCS were excluded due to failed technical quality criteria in one additional participant each.

Free tissue hormones represented a small fraction of the total plasma concentration in all cases (Fig. 4, left column and Fig S29). Consistent with the ambulatory cohort, a daily rhythm was apparent for all hormones except DHEA-S. Pulsatile secretion of aldosterone in plasma was clearly reflected in tissue samples (Fig. 4D). Comparison of the in-laboratory with the ambulatory cohort is shown for illustrative purposes (Fig. 4, right column). There was strong evidence to suggest that concentrations of tissue cortisol, 18-OHF, CCS and aldosterone were lower than in the ambulatory cohort ($p<0.001$).

Data from a single ambulatory participant plotted against the rolling mean (Fig. 4E-H) illustrates the relationship of hormone peaks with individual waking time, which did not align with the mean wake time of the cohort (compare vertical dotted red line with the grey shaded area). Further, for the single individual, pulsatile secretion of tissue aldosterone can be observed (Fig. 4H) – expression of pulsatility is not captured by the underlying rolling mean of aldosterone from all participants.

We then examined the correlation and phase relationships between plasma and tissue steroids. Fig. 5A-E and G shows Z-scored rolling mean concentrations of hormones matched across the two compartments. First, we examined the relationship between ACTH, cortisol, and 18-OHF. Assessing each profile individually, we found strong evidence ($p<0.001$) of correlation not only between ACTH and plasma cortisol (median $r_s = 0.89$ (range 0.78—0.94)), but also ACTH and plasma 18-OHF (median $r_s = 0.91$ (0.74-0.93)), and between plasma cortisol and 18-OHF (median $r_s = 0.97$ (0.96-0.99), Fig. 5A). Next, we examined the same relationships in tissue. There was also strong evidence ($p<0.001$) of a significant correlation between ACTH and tissue cortisol (median $r_s = 0.70$ (0.48-0.77), and between ACTH and tissue 18-OHF (median $r_s = 0.81$ (0.74-0.86), Fig. 5B). However, tissue 18-OHF appeared to be better correlated and better

synchronised with plasma cortisol (median r_s = 0.91 (0.75-0.97), median lag 15 minutes (2-34)), than with tissue cortisol (median r_s = 0.78 (0.56-0.87), median lag 58 minutes (41-60)). Furthermore, the median lag between tissue 18-OHF and tissue cortisol was 45 minutes (1-60).

There was evidence of a moderate correlation of plasma with tissue aldosterone (median r_s = 0.68 (0.22-0.85), Fig. 5E). The correlation of ACTH with aldosterone was overall weak (median r_s = 0.23 (0.1-0.46) in plasma and 0.37 (0.17-0.72) in tissue). However, we found that peaks of ACTH coincided with increases in aldosterone during the overnight period (Fig. S30). The median lag between plasma and tissue aldosterone was 31.5 minutes (23-60).

ACTH was more correlated with plasma CCS (median r_s = 0.87, (0.77-0.94) than with CCS in tissue (median r_s = 0.55 (0.18-0.65). There was moderate correlation of plasma and tissue CCS (median r_s = 0.57 (0.27-0.75). ACTH-associated secretion of CCS overnight was delayed in tissue compared with plasma (Fig. 5G), with a median lag of 60 minutes between compartments.

Cortisol and cortisone were strongly correlated with each other in plasma and tissue (plasma median r_s = 0.94 (0.75-0.95), Fig. 5C, matched tissue median r_s = 0.94 (0.58-0.98), Fig. 5D). Considering CORT to be the sum of cortisol + cortisone (i.e., it's inactive form), we observed that the fraction of cortisol (as a percentage of CORT) was substantially higher in plasma (mean 81 ± 2.5%) than in matched tissue samples (mean 48 ± 7.1%, p<0.001, Fig. 5F). In the larger ambulatory cohort, the mean percentage was 54 ± 5.8%, Fig. 5H). Furthermore, we observed that in tissue profiles, the fraction of cortisol (as a percentage of CORT) varied markedly across the day, on average falling below 50% during the evening (Fig. 5F and H, red shaded areas).

Dynamic markers quantify normal variability in healthy adults

Understanding the physiological and clinical significance of hormonal rhythms requires a shift from qualitative to quantitative descriptions of variability. That is, we needed to devise methods to quantify the limits of "normal" hormonal variation in a single individual, within our population of healthy participants (Fig. 6A). To do this, we searched for dynamic markers (dMs) of "normality" in 24-hour continuous hormone profiles. These dMs are mathematically defined metrics that account for dynamic features of hormonal rhythms unique to each individual (Fig. 6B). A statistical description of dMs within a healthy cohort allowed us to quantify the variability of hormonal rhythms in healthy individuals (Fig. 6C-E). This approach also facilitated comparison of dynamic features specific to sub-populations stratified by sex, age, and other physiological parameters such as body mass index (BMI) and blood pressure (Fig. 6F-J and Fig. S31).

Altogether, we were only able to explain very small amounts of the variability observed between dMs (Fig. S32-38). A small amount of the variance in total DHEA-S was due to increasing age (r^2 = 0.21, p<0.001, Fig. S38), but we found that age accounted for none of the variance in the

morning peak ($p>0.05$) and only very small amounts related to evening nadir concentrations of cortisol ($r^2 = 0.1$, $p<0.001$) and 18-OHF ($r^2 = 0.09$, $p<0.001$, Fig. S33-35. Similarly, we found that BMI only accounted for very small amounts of the variance in the total concentrations of hormones (cortisol $r^2 = 0.08$, $p<0.001$, cortisone $r^2= 0.15$, $p<0.001$, aldosterone $r^2= 0.07$, $p<0.001$, Fig. S33-36). As part of the sampling protocol, each participant had a single blood pressure measurement prior to the commencement of microdialysis sampling. We found no relation between these blood pressure measurements and the total area under the curve, morning peak or evening nadir of aldosterone ($p >0.1$ for all) (Fig. S33-38).

Having established the patterns of healthy variability, we are able to apply our methods in disease, and thereby investigate the importance of altered dynamics in specific endocrine conditions. In Cushing's disease for instance, it is possible to observe in detail disruption of both normal circadian and ultradian rhythmicity (n=1 example profile, Fig. 7A) rather than simply noting increased amounts of cortisol. Similarly, in a n=1 example of primary aldosteronism (Fig. 7B), abnormal secretion of aldosterone in that individual can be quantified by multiple parameters, in addition to total amounts of hormone secreted. Patterns can be better understood using dMs, which provide a more rigorous, quantitative assessment of abnormal hormonal profiles in pathological scenarios. In the case of Cushing's, the area under the curve was higher than in healthy individuals, especially in the evening and overnight observation windows (Fig. 7C). However, additional anomalies were also identified by dMs specific to hormone peak and nadir concentrations and timing (Fig. 7E and G). In the case of the primary aldosteronism example, dMs associated with overnight aldosterone excess and morning peak signalled deviations from their distribution in healthy participants (Fig. 7D, F and G).

U-RHYTHM microdialysis sampling was safe and acceptable

Feedback questionnaires were completed by 212/214 participants revealing that the technique was overall acceptable and well tolerated (Fig. 8). Most participants did not experience any discomfort and were able to continue normal activities during the sampling period. With regards to sleep, 73% agreed it was easy to sleep while wearing the U-RHYTHM collector, 16% were undecided, and the remaining 11% disagreed or strongly disagreed. Most participants (77%) agreed or strongly agreed that they would wear the device again if asked. There were no serious adverse events. In a wider review of all sampling sessions completed using the U-RHYTHM microdialysis system, minor adverse events were reported in 2% (15/649) consisting of local skin reaction to the adhesive dressing (n=7), greater than expected bruising or hematoma (n=6), and discomfort at the probe site (n=2).

DISCUSSION

We have demonstrated the healthy dynamic variation of multiple adrenal steroid hormones in tissue in a large cohort of participants continuing normal daily activities. The microdialysis-based method was very well tolerated across the sampling period when coupled with our prototype fraction collector, known as U-RHYTHM. The resulting data provides us with a dynamic picture of the human *corticosteroidome*, specifically concentrations of active free hormone in tissue, rather than the total of protein-bound plus free hormone found in blood.

This project has yielded several findings. Perhaps the most noteworthy is the establishment of normal 24-hour patterns of adrenal hormones in tissue rather than blood, collected without the need for participant intervention, and during normal daily activity. We achieved this by studying daily profiles in 214 healthy volunteers across a broad age spectrum. Having previously identified the presence of rhythmic free cortisol in subcutaneous microdialysate (*25–27*), here we have further identified and quantified subcutaneous tissue concentrations of cortisone, corticosterone, 18-OHF, aldosterone, cortisol metabolites, and DHEA-S. We found that all these hormones, except for DHEA-S, showed a daily rhythm that coordinated with the rest-activity cycle. This finding is consistent with a system in which the circadian rhythm of adrenal glucocorticoids is primarily modulated by the HPA axis (*12*). In our plasma comparison studies, we found correlation of all the adrenal steroids with ACTH. The strong correlations between plasma and tissue emphasise how important the daily rhythm of the HPA axis must be for normal physiology, as information is conveyed from the circulation to end-organ tissues, where hormones exert their effects (*1*).

We note the substantial inter-individual variability observed in our steroid profiles. This highlights the fact that humans are an outbred population, with every individual possessing a unique daily rhythm determined by both intrinsic mechanisms and behavioral adaptation, as well as different responses to day-to-day hassles. It is these different ‘hassles’, and the individual responses to them that a personalized diagnostic work-up must take into account in the era of precision medicine. Once these points are considered, it helps us to understand why single point blood samples of either glucocorticoid or mineralocorticoid hormones are so difficult to interpret, and to appreciate that there is no such thing as a perfectly ‘normal’ profile, because concentrations can change greatly over a short period of time. Indeed, our data not only reveals the importance of understanding the patterns of hormone secretion across the day but also emphasizes the importance of night-time dynamic change when current hormone testing is not performed. This is likely to be particularly important for the assessment of suspected cases of hormone hypersecretion, where abnormalities on traditional tests for Cushing's disease or primary aldosteronism may be subtle or inconclusive, rather than overtly abnormal.
Our ability to measure multiple steroids simultaneously also allows us to investigate dynamic relationships between hormones. This has revealed interesting differences from what is found in blood. One area of clinical importance is the relationship between biologically active cortisol and

inactive cortisone. It is well established that glucocorticoid action on target tissues is determined in part by the two isozymes of 11β-hydroxysteroid dehydrogenase (11β-HSD) that catalyze interconversion of cortisol and cortisone (31). Our data clearly shows that the ratio of cortisol to cortisone is different in subcutaneous tissue to that in blood (32). This suggests both that the activity of tissue 11-bHSD has an important effect on the availability of cortisol at tissue glucocorticoid receptors, and that the simple measurement of plasma cortisol may be misleading. Clearly, it will be of great interest to explore the tissue specificity of this finding by conducting microdialysis studies in different tissues such as skeletal muscle or specific areas of brown and white adipose tissue. Our data also shows that the cortisol to cortisone ratio varies across the day –a finding that may prove to be both of clinical and therapeutic importance with the development of new 11-bHSD1 inhibitors (33). A better understanding of tissue cortisol regulation is likely to prove important across many areas of medicine including metabolic disorders and obesity, cardiovascular disease, hypertension, inflammatory diseases and most recently in Alzheimer's disease, in all of which abnormalities of HPA function have been described (13).

Another notable finding was the close relationship between cortisol and 18-OHF in both plasma and microdialysate. Previous studies have placed ACTH as the primary driver of 18-OHF secretion (*34*) and our data, for the first time characterising detailed 24-hour profiles of the hormone, is entirely consistent with this. Furthermore, our study shows tissue 18-OHF was both a better predictor of plasma cortisol and phase advanced with respect to tissue cortisol. This has interesting implications for future studies – tissue 18-OHF may be the hormone that provides the best information about the temporal dynamics of adrenal cortisol, separate from any local tissue effects on concentrations of cortisol and its metabolite cortisone.

The accepted wisdom is that the synthesis of 18-OHF from cortisol requires both aldosterone synthase from the zona glomerulosa and 17α-hydroxylase from the zona fasciculata (*34*). This concept certainly fits with increases in 18-OHF reported in primary aldosteronism (*35*), with the caveat that in comparison with aldosterone, 18-OHF is more responsive to ACTH than plasma renin activity (*36*). Our data certainly are more in line with a primary regulation by ACTH –at least in our healthy participants on an unrestricted salt intake, and without a diagnosis of hypertension. An additional factor to bear in mind in interpreting our data is that corticosterone, which is synthesised directly from progesterone and is the direct precursor of aldosterone (Fig. 1), also has a very close relationship with the circadian pattern of both cortisol and 18-OHF. Recent data has suggested that this hormone may have distinct tissue-specific glucocorticoid effects in humans, related to its ABCC1 transporter affinity for which ACTH dependence would seem appropriate (*37*).

Our ability to measure multiple steroids in each sample enables exploration of temporal associations of changes in glucocorticoid and mineralocorticoid hormones. Of particular interest is the relationship of cortisol and aldosterone. It is generally accepted that in normal healthy

individuals such as the participants in this study, the renin-angiotensin system is the major regulator of aldosterone secretion. This, however, is difficult to reconcile with evidence showing that plasma aldosterone correlates better with plasma cortisol than with plasma renin activity (*16*). It is becoming increasingly clear that there are multiple factors involved in the regulation of aldosterone including not only renin activity and ACTH (*7*, *15*), but also sleep structure (*16*, *17*, *38*) and potassium intake (*19*). It is important to note that our participants were all on unrestricted diets, without any specific control of salt intake. Although this could explain differences in cortisol and aldosterone regulation between subjects during the day, one factor in common to almost all participants was the coordination of the ACTH dependent anticipatory pulse of cortisol with a pulse of aldosterone prior to awakening. Further exploration of these relationships in subjects on different sodium intakes should help characterise whether the adrenal glomerulosa cells show differential sensitivity to ACTH and plasma renin activity in different states of fluid balance. A better understanding of the normal patterns of aldosterone secretion should also aid both in our diagnosis of primary aldosteronism and hopefully the differentiation of patients with unilateral primary aldosteronism due to Conn's adenoma and those with bilateral adrenal hyperplasia.

Understanding variability is important to assess the limits within which healthy individuals can display a range of physiological hormone rhythms. Our dynamic markers (dMs) allow us to shift from a qualitative to a quantitative description of variability. Because they are mathematically defined, dMs constitute an unambiguous method to describe healthy hormonal rhythms and facilitate further analysis through non-stationary statistics and machine learning techniques. (*39*) In our analysis, it is interesting that we found only a few factors likely to influence healthy hormone concentrations, and none were profound. There is, however, certainly scope for a more detailed analysis of the interindividual variation observed in the population, including the alignment of rhythms with respect to sleep rather than clock time, geographical and seasonal differences, and the degree to which daily rhythms may or may not be stable within a single individual across time.

Our dMs allow stratification of normality across sub-populations (for example by sex, age, BMI, etc.), and the same approach can also be generalised to quantify hormonal misalignment arising from behavioural and environmental factors (such as sleep, light exposure, meal content and timing, smoking habits, etc.) (*39*). The combination of high time-resolution continuous hormone sampling with non-invasive wearable devices and computational analysis techniques has the potential to support clinical diagnosis and disease management according to individual physiotypes (*40*). Because of differences in disease presentation and population heterogeneity, specific dM values will vary between individuals. This represents a challenge in identifying what set of dMs are key to support specific diagnoses, one that needs to overcome the limitations of linear regression and conventional statistical methods. Therefore, integrating dMs into machine

learning analysis pipelines that account for sources of heterogeneity will be instrumental to support personalised interventions.

In the future, in addition to supporting diagnosis and management of hormone excess, this technology of dynamic tissue hormone measurement will also allow us to assess hormone replacement therapy in patients going about their normal daily activities - which is of course what we really want to know. Better replacement therapeutics are clearly needed (*41*) and it will be very important to assess the potential benefits of these preparations using ambulatory 24-hour monitoring to ensure patients have optimised therapy with long-term efficacy, safety, convenience, and affordability. An important aspect of this will be ensuring overnight profiles match the physiologically normal anticipatory increase in cortisol that occurs prior to awakening (*12*).

This study represents proof-of-principle that the U-RHYTHM method can be used at scale to measure multiple hormones dynamically. However, there are some limitations to the current work that need to be acknowledged. It is apparent that the major changes in hormones occur around the time of waking and future work should employ more objective measures of sleep timing (*42*) in addition to subjective self-report. Additionally, microdialysis necessarily provides an estimation of the local tissue hormones rather than absolute concentration (*43*) and therefore it is possible that our microdialysis measurements are an underestimate of the true tissue concentrations of free hormones. We minimised methodological variability by using a consistent sampling protocol and high-performance measurement system, suggesting that differences in hormone concentrations between healthy participants are likely to be related to multiple factors, most of which are difficult to quantify. Further analysis aimed at identifying sources of interindividual variability should include aligning profiles by wake time, and stratification of profiles according to season and latitude. One other relevant factor relates to fat mass, which has previously been shown to influence the microdialysate recovery of pharmaceutical compounds (*44*) and evidence suggests that adipose also has an impact on tissue cortisol metabolism in obesity (*45*). Last, our study did not include participants with obese BMI, and this should be a priority for future research.

In conclusion, we have demonstrated how an interdisciplinary approach of a novel ambulatory sampling system, liquid chromatography-mass spectrometry technology and mathematical analytical techniques have allowed us to establish the normal dynamics of the 24-hour tissue *corticosteroidome*. As well as providing data on the physiology and dynamics of tissue steroids, we have provided the ‘normal’ reference values on which we can define changes associated with adrenal disease, alongside a powerful methodology to improve personalised replacement therapy.

MATERIALS AND METHODS

Experimental design. The study aimed to define daily variation in the levels of cortisol, aldosterone and their metabolites in healthy participants carrying out normal daily activities, including during uninterrupted sleep. To do this we designed a portable automated microdialysis-based fraction collector enabling continuous sampling of hormones in subcutaneous tissue. Over 24 hours, 72 samples were obtained for each participant representing a 20-minute sampling frequency. Corticosteroid levels were measured in each sample by liquid chromatography tandem mass spectrometry. The resulting curves were analysed by time series analysis methods. Healthy volunteer data was included from the Dynamic Hormone Diagnostics (ULTRADIAN) observational cohort study (NCT02934399). Participants were recruited from the general population between 2016-2020 to study sites in Bergen (Norway, Regional Ethics Committee West 2015/872), Bristol (UK, South West – Frenchay Research Ethics Committee IRAS 181429), Athens (Evangelismos Hospital 208/20-10-2015, Greece), and Stockholm (Sweden, Swedish Ethical Review Authority 2016/1463-31/4).

Participant recruitment and screening. Males and females aged 18-68 were eligible for inclusion. Exclusion criteria were: BMI <16 or ≥ 30 kg/m^2, use of estrogen-containing oral contraceptive medication within the past 6 weeks (plasma comparison studies only), pregnancy or lactation, regular prescribed medicine, medical conditions active within the past 3 months, any history of endocrine disorder, use of oral, inhaled, parenteral, or topical glucocorticoids within past 3 months, regular alcohol intake greater than 26 units per week, any use of potentially interfering herbal or over-the-counter supplements within 14 days, abnormality of screening blood tests of renal function, haemoglobin, white cell count and differential, fasting glucose and glycated haemoglobin (HbA1c), liver function tests, thyroid stimulating hormone (TSH) and free T4, and tobacco smokers (plasma comparison studies only). Immediately prior to the start of each sampling session, participants provided a urine sample to exclude illicit substance use (SureScreen Diagnostics) and in females <50, pregnancy (VWR International Ltd).

Pre-study conditions. Participants were asked to maintain a regular bedtime, abstain from alcohol and all medication, and to avoid any strenuous physical activity for 48 hours prior to the sampling session. Participants were asked to complete an activity diary, including records of food type and timing, sleep timing and subjective quality, commencing 24 hours prior to the sampling session.

U-RHYTHM microdialysis system. The concept and general mechanism of action of this sampler has been described previously (*25*), with substantial further design and performance optimisation for the current studies carried out by our design and manufacturing partner, Designworks Windsor. Briefly, a 20kDa cut-off linear microdialysis catheter (membrane length 30mm, mDialysis) was placed perpendicular to the midline in periumbilical subcutaneous tissue, flushed, and then perfused at 1µL/min (107 microdialysis pump, mDialysis) with sterile isotonic fluid (T1 perfusion fluid, mDialysis). The outlet of the probe was adapted to connect to our fraction collector (U-RHYTHM, DesignWorks) using 20cm of FEP tubing, allowing the

automated collection and storage of individual samples every 20 min. The pump and sampler assembly were placed in a flexible waist belt (LimberStretch, Egham).

U-RHYTHM ambulatory sampling protocol. After placement of the U-RHYTHM microdialysis system, participants were permitted to continue most normal activities except for vigorous physical exercise (which could potentially dislodge the probe or sampling connections) and submersion in water (as the pump and U-RHYTHM device are not waterproof). There were no restrictions placed on diet. Sleep/wake times were determined by the participant.

Comparative study of plasma with microdialysate. Participants arrived fasted in the morning of the sampling day. The microdialysis probe was placed in the right periumbilical subcutaneous tissue and connected to the fraction collector according to standard procedure. An 18g venous cannula was placed in an antecubital vein of the non-dominant arm and connected to an automated blood sampling system allowing the collection of individual samples every 20 min (*10*). During the procedure, participants remained on the study bed, except for visits to the toilet. A standardised set of meals providing 2225kCal (83g protein, 273g carbohydrate, 83g fat, 27g fibre) as breakfast (08:00), lunch (13:00), dinner (19:00) and a snack (22:00) was provided with free access to water, tea, and coffee. Room brightness was reduced to <10 lux as measured at the angle of gaze between 19:30-23:00 and 07:00-09:00. Participants were asked to dim electronic devices and activate 'night modes' during these times. Room brightness was reduced to <5 lux during the sleep period (between 23:00-07:00).

Assessment of U-RHYTHM acceptability. On completion of sampling, participants completed a feedback questionnaire regarding their experience with the U-RHYTHM microdialysis system (see Supplementary Materials and Methods).

Assays. Microdialysis samples were retrieved from the U-RHYTHM collector following the end of each sampling session. The whole volume of each sample was decanted into 300µL polypropylene sample vials (Microbiotech/se) and then frozen at -80°C prior to analysis. Aliquots of the plasma were frozen at -20°C until the end of the experiment and then at -80°C until analysis. Immunoassay for plasma adrenocorticotropic hormone (ACTH, IMMULITE 2000, Siemens Healthcare GmbH) and liquid chromatography-mass spectrometry measurement of adrenal steroids was performed at the Ultradian Analysis Platform, Core Facility for Metabolomics, Department of Clinical Science, University of Bergen, Norway. Measurement of adrenal steroids was achieved using an assay developed and expanded from a previously published method (*46*, *47*) and further optimised for measurement of free steroids in microdialysis samples. Sample preparation process was automated using Hamilton Star (Hamilton Robotics). Microdialysate samples (10 µl) or serum (45 µl) samples were transferred to a 96-well plate with borosilicate glass inserts containing a mixture of isotopically labelled internal standards in 50 µl H_2O:MeOH (60:40). The volume of each microdialysate sample was

measured using pressure-based liquid detection and the aspirated volume was algorithmically reduced to a minimum of 5 µL when sample volume was less than 15 µL. The plate was then vortex mixed for 1 minute, and 420 µl ethyl acetate:hexane (80:20) was added and mixed by repeated aspirate-dispense cycles. The phases were allowed to settle for 10 minutes before 300 µl of the organic extracts were transferred to a 96-well plate. Samples were evaporated at 50°C under N_2 flow and reconstituted in 50 µl (microdialysate) or 100 µl (serum) H_2O:MeOH (60:40). The processed extracts (20 µL for microdialysate, 5 µL for serum) were analysed by liquid chromatography triple quadrupole mass spectrometry on a Waters Xevo TQ XS I-class instrument equipped with UniSpray ion source. For details on methodology of chromatographic separations and mass spectrometry, see Supplemental information. The panel included 21 glucocorticoid, mineralocorticoid and androgen hormones, precursors and metabolites and 1 synthetic glucocorticoid (dexamethasone) (Table S3).

Study participants. Ambulatory microdialysis sessions were successfully completed in n=228 unique participants. Of these, 14 profiles were excluded: n=6 due to analytical issues, n=6 on failed inclusion criteria (BMI exceeded n=2, consumption of alcohol or glucocorticoids n=2, previous endocrine diagnosis n=1, recent night shift work n=1), and n=2 due to incomplete profiles, leaving n=214 unique individual profiles available for analysis. In this cohort, there were n=98 male, the age range was 20-68 years (mean 42.5 ± 14.1), mean BMI 23.5 ± 2.7 $kg.m^2$, median self-reported sleep onset times 23:28 hrs ± 58 min and final wake 7:04 hrs ± 76 min. Comparative sampling sessions were completed in n=7 participants (n=1 female, mean age 29.7 years (24-44), mean BMI 25.4 kg/m^2 (22.3-27.8)). Technical issues with the automated blood sampling system resulted in an incomplete blood sample collection for participant 3 (56/72 samples) and 4 (69/72 samples).

Statistical methods. A toolkit for the analysis of hormonal time series data was developed by the authors and implemented in Python (ver. 3.8.12) using numeric and data analysis libraries NumPy, SciPy, Pandas, CosinorPy and plotting libraries Matplotlib and Seaborn. A threshold for significance was set at $p<0.05$.

Each dynamic hormone profile consisted of a regularly sampled time series. Because microdialysis fluid was collected continuously, the time for each individual sample was taken as the midpoint of each collection period (e.g., microdialysate collected between 09:01-09:20 was labelled 09:10, between 09:21-09:40 was labelled 09:30, and so on). Since the sample times in plasma were offset by a maximum of 10 min with respect to the microdialysate (sampled every 20 min), the latter was run through a cubic spline interpolation algorithm for 10 min up-sampling to facilitate comparison between hormone profiles across both compartments. In the case of missing data, the maximum number of consecutive samples that could be interpolated was set to 3. If >50% of data points were missing in a profile, that profile was excluded from any further correlational analysis.

Time-lagged cross-correlation (TLCC) analysis was performed by incrementally shifting (lagging) one series relative to the other and repeatedly calculating the cross-correlation between each profile. Using a lag step of 1 min, the TLCCs were calculated to a maximum lag of 180 min from the zero-lag centre. Since the sample populations do not follow a normal distribution, the strength of the TLCC was evaluated using Spearman's rank r_s (where $r_s = 1$ is perfect correlation and $r_s = -1$ is perfect anticorrelation).

For a dynamic examination of how TLCC changes during the day, we calculated the rolling window time-lagged cross-correlation (RWTLCC) (*48*). A rolling window aggregation was performed on the standardised (Z-scored) time series using a Gaussian window of 60 min, generating a normally distributed mean value at each time point. The Gaussian means were then run past a cross-correlation function. The strength of the correlation was assessed using Pearson's correlation coefficient r_p (where $r_p = 1$ is perfect correlation and $r_p = -1$ is perfect anticorrelation).

Non-linear cosinor regression analysis was used to detect and quantify circadian and ultradian rhythmicity in 24-hour hormone profiles, including estimations of acrophase and mesor (*30*). We used a population-level 3 component cosinor model with 24-hour standardised (Z-scored) hormone time series data. No data imputation was used, but profiles with the largest continuous data gaps were excluded to avoid spurious fits: cortisol (0), aldosterone (30), 18-OHF (1), corticosterone (30), cortisone (0), aTHF (40) and THF (20). Linear regression was used to quantify associations between measured analytes per time point and population characteristics including participant metadata and dynamic markers.

For comparison between groups non-parametric (Mann-Whitney) and ordinary least squares regression models were used with Bonferroni correction for multiple comparisons, as indicated.

The dynamic markers (dMs) were defined as: AUC: area under the curve (cumulative sum) during intervals 9-15 hrs, 15-21 hrs, 21-3 hrs and 3-9 hrs with periodic boundary conditions; evening nadir (EN_{nadir}): the minimum hormone concentration within the 18-6 hrs interval; morning peak (AM_{peak}): the maximum hormone concentration within the 3-12 hrs interval; peak to nadir ratio (AM_{peak} / EN_{nadir}); overnight secretion density ($D_{peak} = AM_{peak} / AUC_{3\text{-}12\ hrs}$); evening nadir time ($EN_{time}$); morning peak time ($AM_{time}$); and overnight secretion start time (OSS_{time}): time at which concentration reaches 20% of the range from EN_{nadir} to AM_{peak}. Statistical distributions of dMs were obtained using Seaborn's scott kernel density estimator.

Metadata from participant activity diaries and feedback questionnaires were managed using REDCap electronic data capture tools hosted at University of Bristol (*49*, *50*). Anthropometric

and other data related to the sampling sessions was managed using an in-house electronic database solution hosted by University of Bergen.

List of Supplementary Materials

Methods and Materials

Figures S1 to S38

Tables S1 to S7

MDAR reproducibility checklist

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Competing interests:
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All other authors declare that they have no competing interests.

Data and materials availability:

Data relating to the paper are deposited in the public database UiB Open Research Data available at https://doi.org/10.18710/5TW8YF. The data consists of anonymized participant metadata, time point measurements of hormone concentrations for each individual and metadata about measurement performance in the form of comma separated value (CSV) files. A data dictionary describing the fields is included.

Figure and table captions

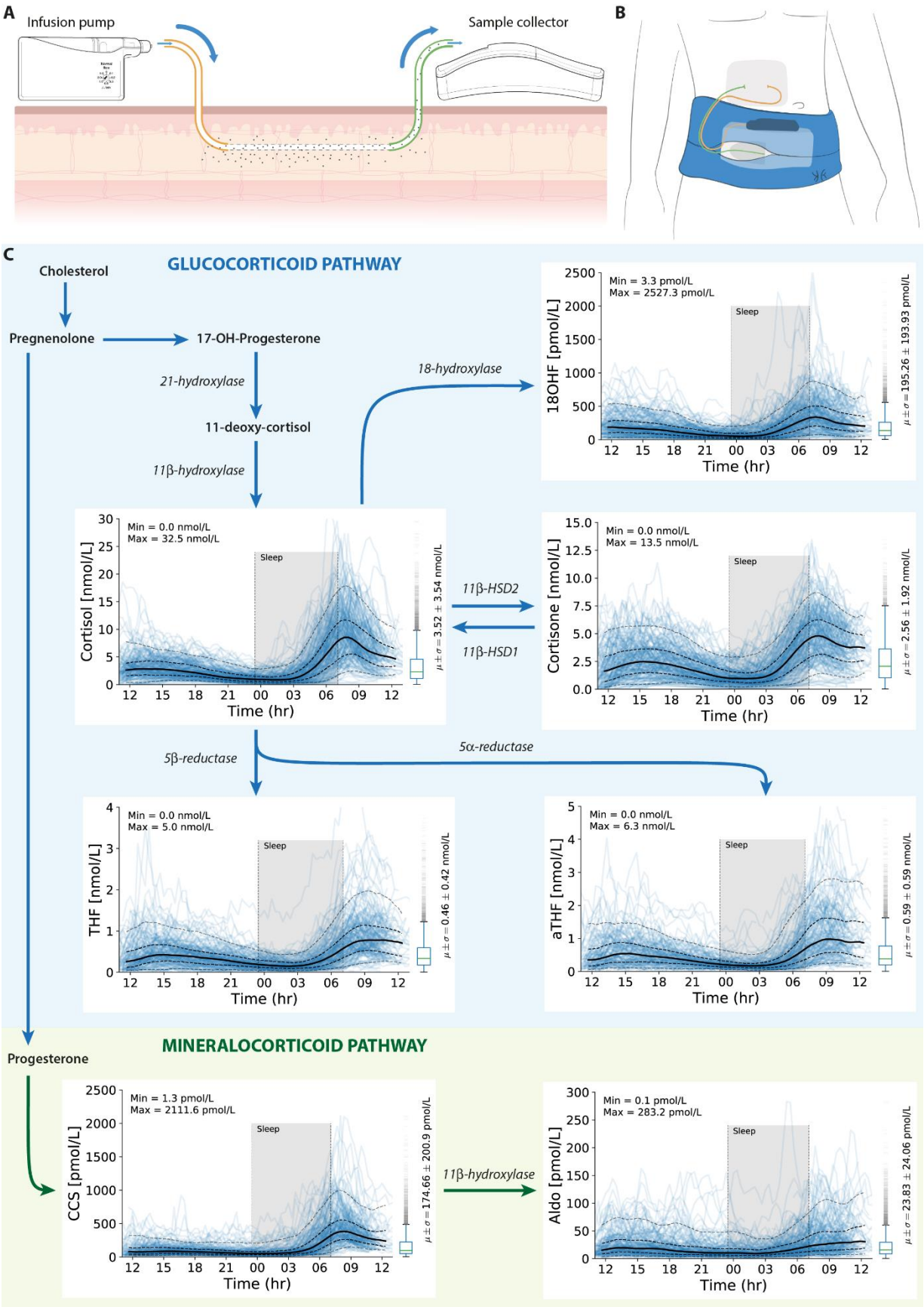
A
Infusion pump
Sample collector
B
C
GLUCOCORTICOID PATHWAY
Cholesterol
Pregnenolone
17-OH-Progesterone
21-hydroxylase
11-deoxy-cortisol
11β-hydroxylase
18-hydroxylase
11β-HSD2
11β-HSD1
5β-reductase
5α-reductase
Progesterone
MINERALOCORTICOID PATHWAY
11β-hydroxylase
18OHF [pmol/L]
Min = 3.3 pmol/L
Max = 2527.3 pmol/L
$\mu \pm \sigma = 195.26 \pm 193.93$ pmol/L
Cortisol [nmol/L]
Min = 0.0 nmol/L
Max = 32.5 nmol/L
$\mu \pm \sigma = 3.52 \pm 3.54$ nmol/L
Cortisone [nmol/L]
Min = 0.0 nmol/L
Max = 13.5 nmol/L
$\mu \pm \sigma = 2.56 \pm 1.92$ nmol/L
THF [nmol/L]
Min = 0.0 nmol/L
Max = 5.0 nmol/L
$\mu \pm \sigma = 0.46 \pm 0.42$ nmol/L
aTHF [nmol/L]
Min = 0.0 nmol/L
Max = 6.3 nmol/L
$\mu \pm \sigma = 0.59 \pm 0.59$ nmol/L
CCS [pmol/L]
Min = 1.3 pmol/L
Max = 2111.6 pmol/L
$\mu \pm \sigma = 174.66 \pm 200.9$ pmol/L
Aldo [pmol/L]
Min = 0.1 pmol/L
Max = 283.2 pmol/L
$\mu \pm \sigma = 23.83 \pm 24.06$ pmol/L
Sleep
Time (hr)
12 15 18 21 00 03 06 09 12

Figure 1. Dynamic profiles of adrenal hormones in ambulatory healthy volunteers. A) The U-RHYTHM sampling system consists of a miniature infusion pump perfusing a linear microdialysis catheter (mDialysis, Sweden) placed in abdominal subcutaneous tissue and connected to a portable sample collector. **B)** The U-RHYTHM system is worn around the waist, permitting ambulatory sampling during almost all normal daily activities, including sleep. **C)** Daily adrenal steroid hormone profiles collected simultaneously in healthy volunteers (n=214, blue lines) using the U-RHYTHM method. Continuous black lines indicate rolling mean concentrations; dashed lines indicate 5, 25, 75 and 95% quartiles (bottom to top). Box plots

indicate stationary statistics (Table 1). 18-OHF =18-hydroxycortisol, THF = tetrahydrocortisol, aTHF = allo-tetrahydrocortisol, CCS = corticosterone, Aldo = aldosterone.

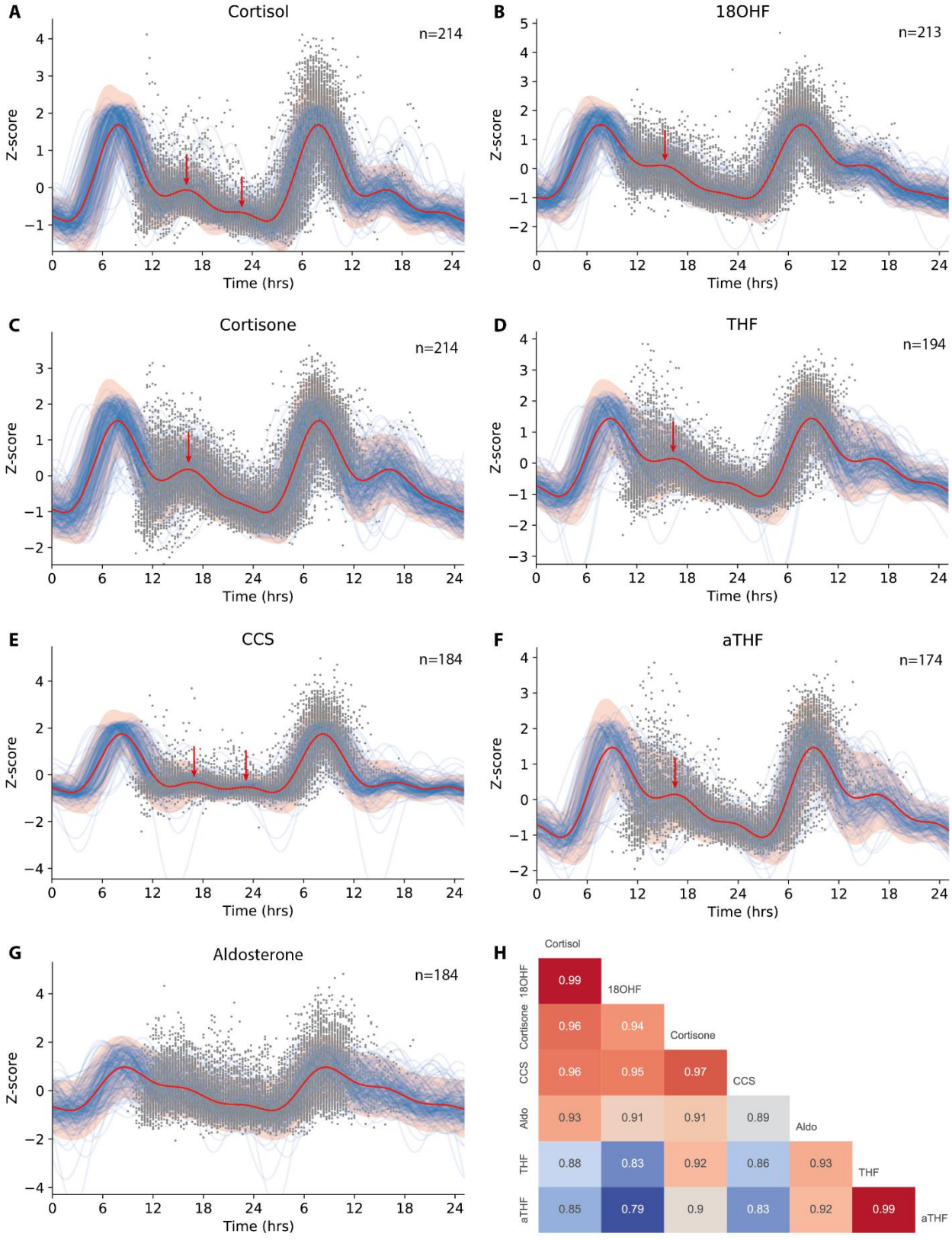

Figure 2. Rhythmometry analysis of 24-hour hormone time series data. A total of 1498 profiles (7 hormones x 214 participants) were examined. **A-G)** Population-level multiple component cosinor fits of standardised (Z-scored) hormone time series data for cortisol (A), 18-hydroxycortisol (18OHF) (B), cortisone (C), tetrahydrocortisol (THF) (D), corticosterone (E), allo-tetrahydrocortisol (aTHF) (F), aldosterone (G). Gray points are individual data points, blue lines indicate individual cosinor fits. The population fit (solid red), standard deviation (shaded red area) and secondary peaks (red arrows) are also shown. **H)** Spearman cross-correlation matrix of hormone data computed from the median concentration of each hormone at each 20-minute time point.

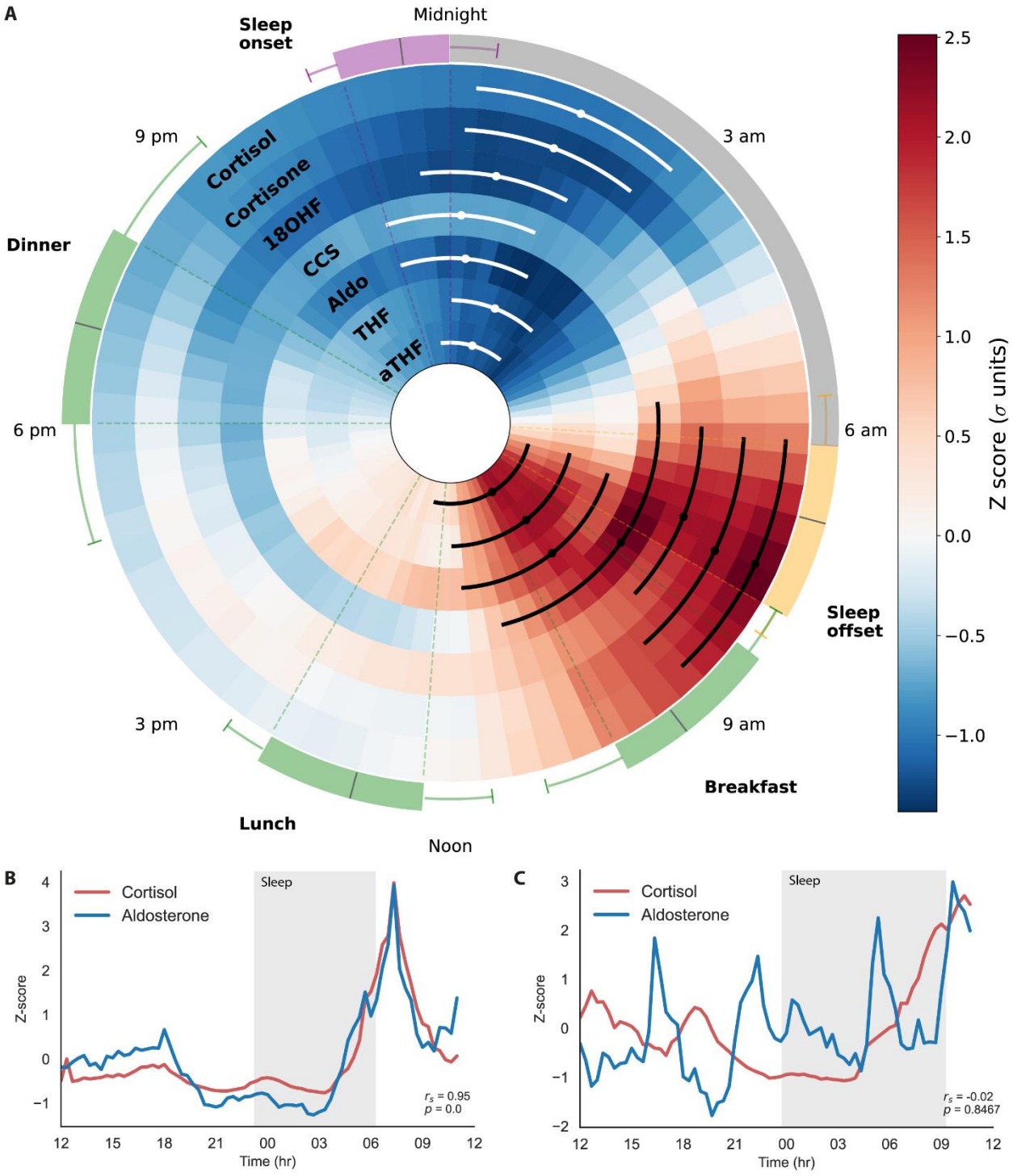

Figure 3. Daily variability of hormonal and behavioural parameters in healthy individuals. **A)** Standardised (Z-scored) rolling mean concentrations of 7 adrenal steroids in n=214 healthy individuals plotted in a clockwise circular heatmap. Coloured cells represent consecutive 20-minute periods. Arc lengths account for median ± SD of the hormonal evening nadir (white) and morning peak (black) for the cohort. Outer rim boxplots account for population variability in the times for sleep onset (purple), sleep offset or awakening (yellow) and meals (green). **B-C)**

Simultaneous 24-hour cortisol and aldosterone profiles in two separate individuals, illustrating the extreme but unusual variability in some hormone profiles.

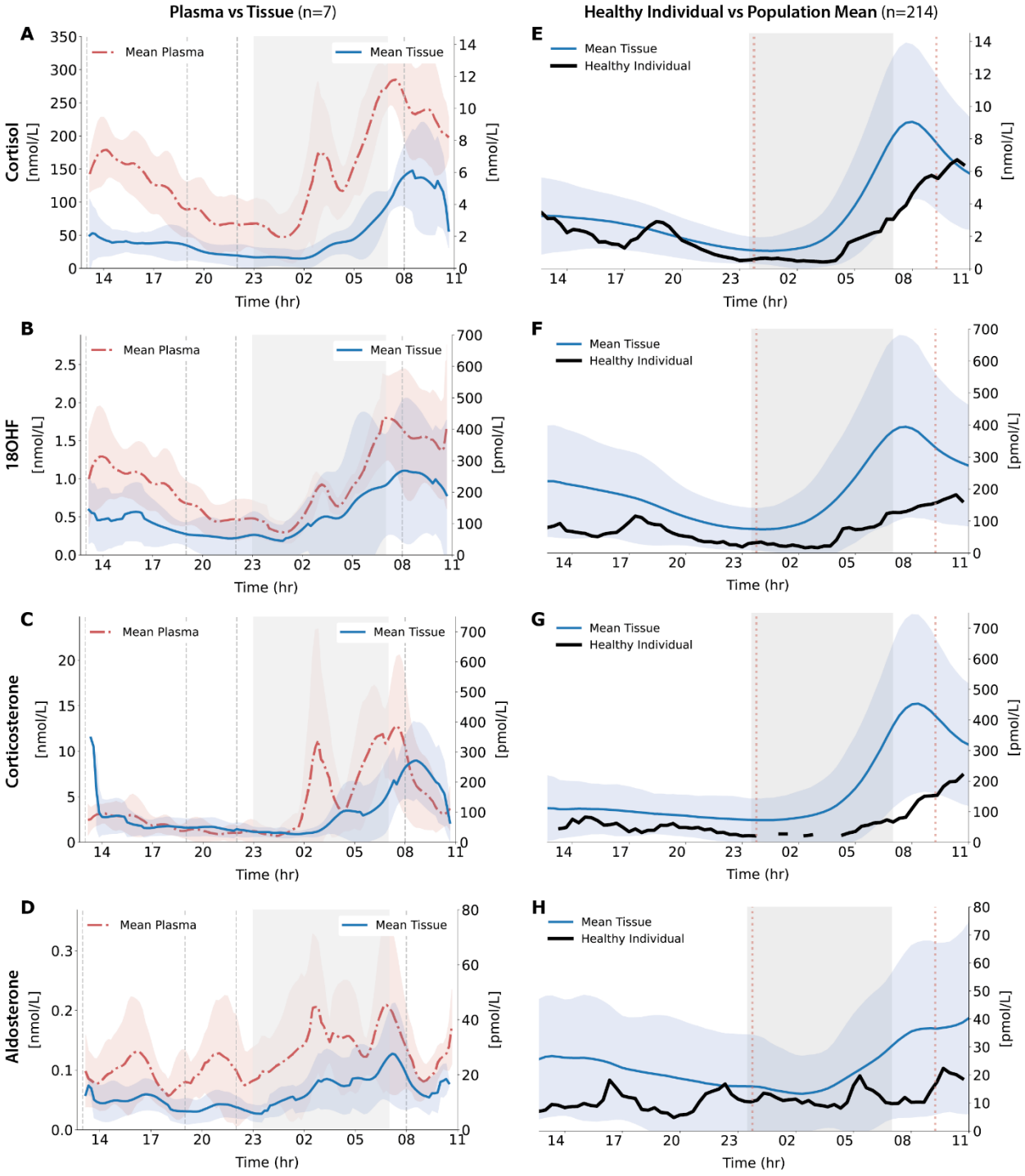

Figure 4. Rolling mean comparisons of tissue and plasma steroids in ambulatory and supine-laboratory sampled healthy participants. Rolling mean concentrations of **A)** cortisol **B)** 18-OHF, **C)** corticosterone and **D)** aldosterone measured simultaneously in n=7 plasma (red dashed lines) and tissue (blue lines) participants in supine laboratory conditions. Rolling mean

tissue concentrations of hormones **E)** cortisol, **F)** 18-OHF, **G)** corticosterone and **H)** aldosterone as measured in n=214 ambulatory participants (blue lines). Cohort sleep periods are shown as shaded grey boxes. Sleep was scheduled between 2300-0700 in the laboratory cohort (left column) while for the ambulatory cohort the mean time between sleep onset and offset is shown (right column). Rolling mean (lines) and standard deviation (shaded areas) of hormones are indicated, whereas data from a single healthy individual is shown as an example (solid black line) with that individual's sleep period marked by vertical dotted red lines.

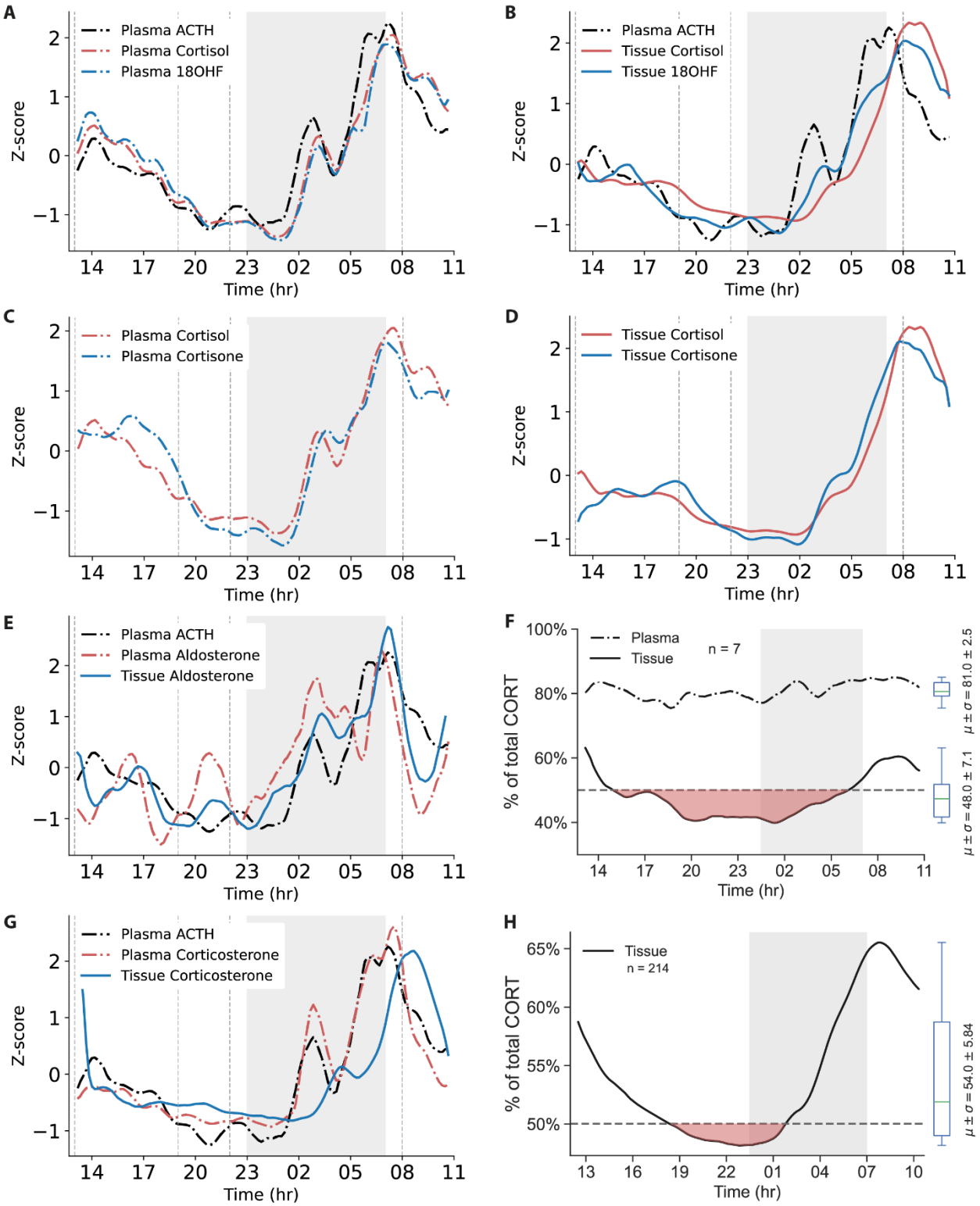

Figure 5. Dynamics of ACTH and adrenal steroid hormones collected simultaneously in plasma and tissue of non-ambulatory and ambulatory participants. A) ACTH, plasma cortisol and 18-hydroxycortisol, **B)** ACTH, tissue cortisol and 18-hydroxycortisol, **C)** plasma cortisol and cortisone, **D)** tissue cortisol and cortisone, **E)** ACTH, plasma and tissue aldosterone,

F) ACTH, plasma and tissue corticosterone, expressed as standardised (Z-scored) rolling mean concentrations of 24-hr hormone profiles in (n=7) supine laboratory-sampled participants. **G)** Tissue cortisol dynamics as percentage of total CORT (cortisol + cortisone) in the ambulatory cohort (n=214). **H)** Tissue and plasma cortisol dynamics as percentage of total CORT (cortisol + cortisone, in each respective compartment) in the laboratory cohort (n=7). Average sleep intervals (shaded grey area), mealtimes (dashed vertical lines), 50% ratios (dashed horizontal lines) and stationary distributions (boxplots) are also indicated.

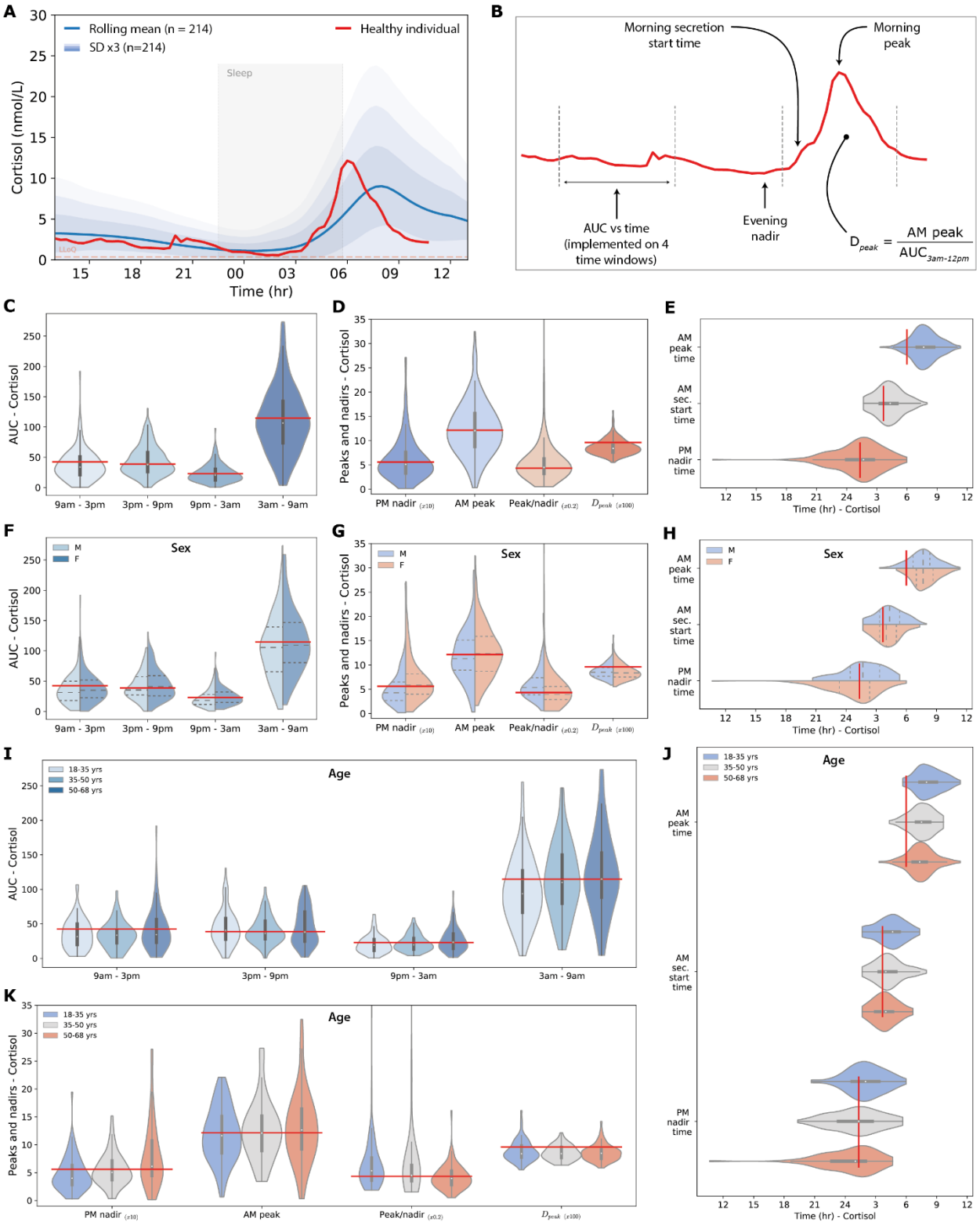

Figure 6. Dynamic markers quantify variability across subpopulations, example of cortisol-specific dynamic markers in a cohort of 214 healthy participants. A) Cortisol profile of a single individual plotted against the background of the healthy cohort (rolling mean ± 3 SDs).

The shaded grey area indicates that individual's sleep interval. **B)** Dynamic markers (dMs) accounting for the dynamic properties of continuous hormone profiles (see Methods). **(C)** Area under the curve (AUC) distributions for four 6-hour long time windows. **D)** Evening nadir, morning peak, peak to nadir ratio and secretion density (D_{peak}) distributions. **E)** Evening nadir time, morning secretion start time and morning peak time distributions. **F-H)**-Distributions of dMs AUC, peak and nadir levels and corresponding peak and nadir times for cortisol, stratified by sex. **I-K)** Distribution of dMs AUC, peak and nadir levels and corresponding cortisol peak and nadir times for cortisol, stratified by age group. Dashed lines and boxes within violin plots indicate 25, 50 and 75% quartiles, whiskers extend from 5 to 95% quartiles. Straight red lines indicate the dM values of a single individual profile (**A**) against the dM distributions in the healthy cohort.

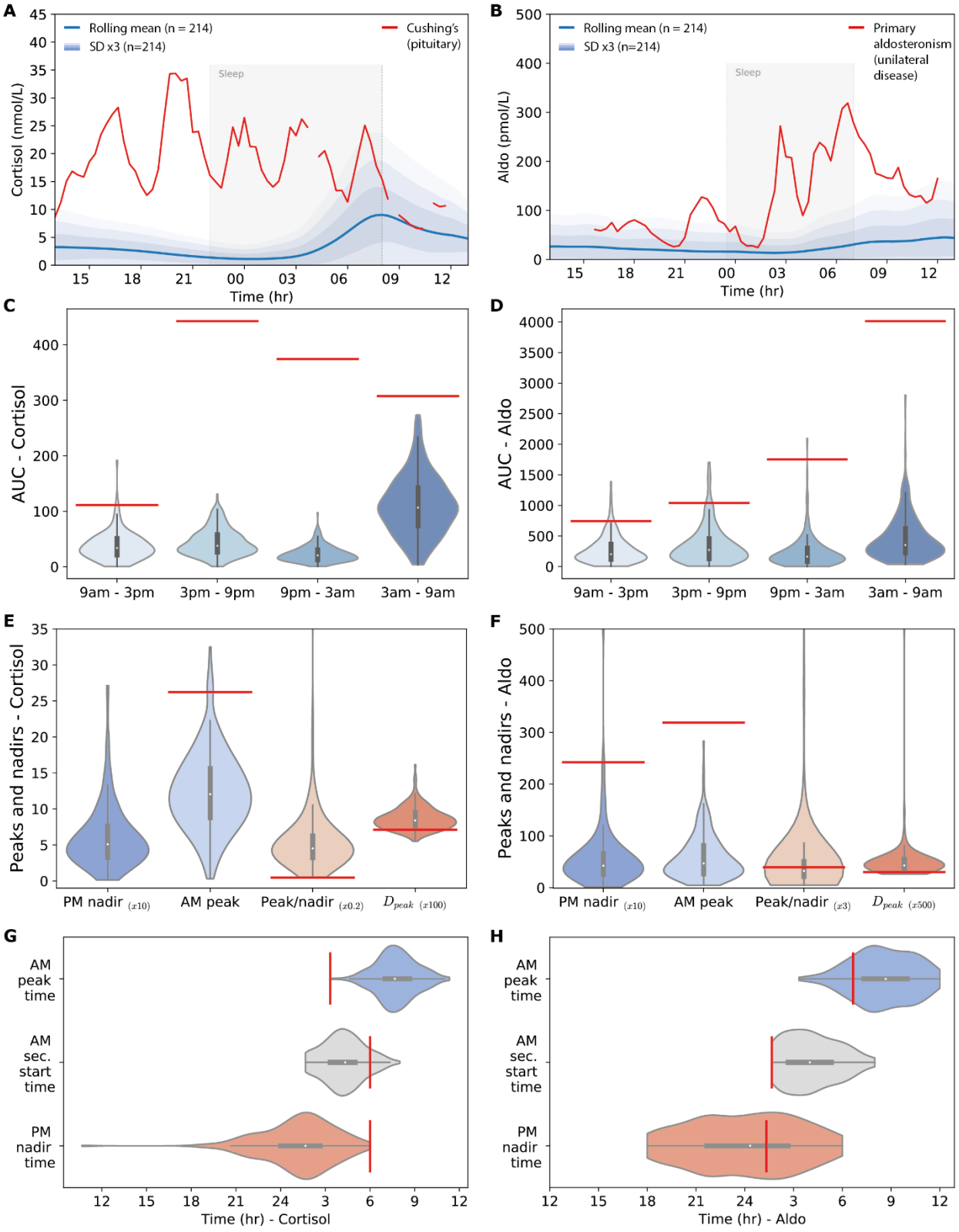

Figure 7. Dynamic markers can help distinguish pathological hormone profiles from normal healthy variability. A) Cortisol profile of a single patient with pituitary Cushing's

plotted against the background of a healthy cohort of 214 participants (rolling mean ± 3 SDs). **B)** Aldosterone profile of a patient with primary aldosteronism plotted against the background of a healthy cohort (rolling mean ± 3 SDs). Shaded grey areas indicate individual sleep intervals. Dashed vertical lines indicate medication times. **C-H)** dM values (straight red lines) from a patient with Cushing's (A) and a patient with primary aldosteronism (B) plotted against cortisol and aldosterone dM distributions. Note that the dM value of the PM nadir for cortisol (E) exceeds the upper limit of the axis and therefore is not visible.. Boxes within violin plots indicate 25, 50 and 75% quartiles, whiskers extend from 5 to 95% quartiles.

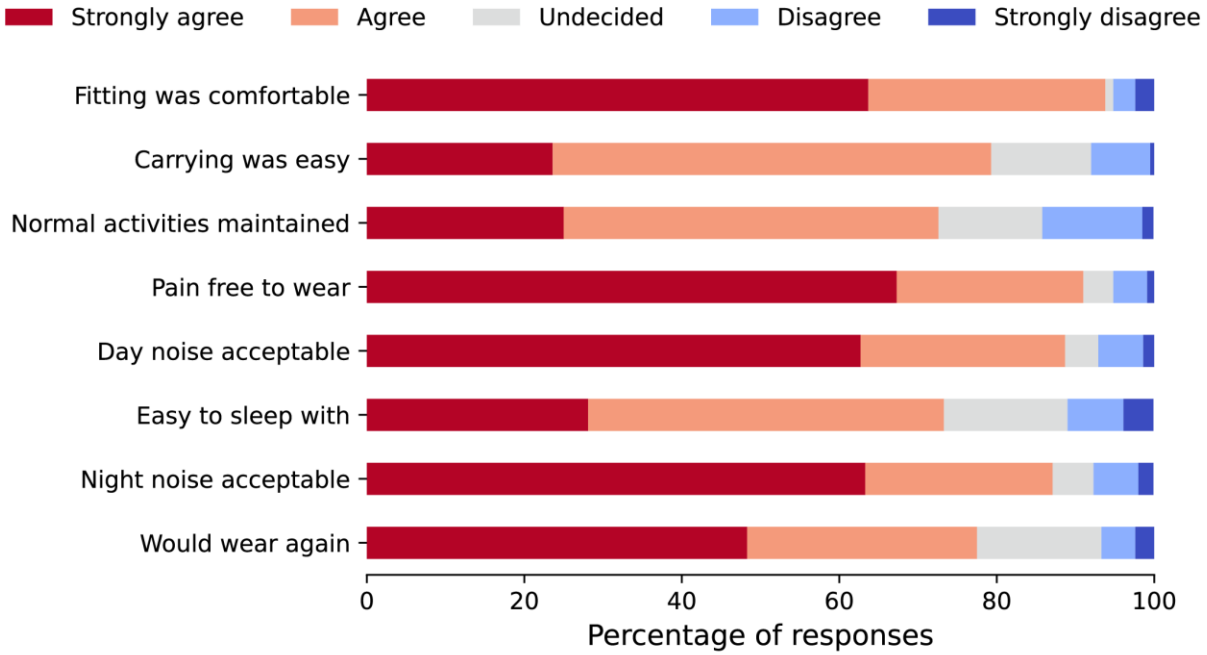

Figure 8. The U-RHYTHM microdialysis system was acceptable to participants. Responses from n=212 participants indicate that the U-RHYTHM microdialysis is well tolerated and allows most normal daily activities to continue.

Table 1. Stationary statistics of hormone concentrations measured in U-RHYTHM microdialysis samples (total n=15408). The discrepancy between mean and median suggests hormones are not normally distributed across all time points. See box plots in Fig. 1C and main text for abbreviations.

	Mean (Median)	SD of mean	Min	Max
Cortisol (nmol/L)	3.52 (2.31)	3.54	0.01	32.47
Cortisone (nmol/L)	2.56 (2.06)	1.92	0.03	13.47
18-OHF (pmol/L)	195.26 (137.57)	193.93	3.26	2527.28
CCS (pmol/L)	174.66 (98.29)	200.9	1.3	2111.61
Aldosterone (pmol/L)	23.83 (15.83)	24.06	0.11	283.2
aTHF (nmol/L)	0.59 (0.38)	0.59	0.0	6.35
THF (nmol/L)	0.46 (0.34)	0.42	0.0	4.96
DHEA-S (nmol/L)	16.15 (14.28)	10.0	2.51	82.48

Table 2. Characterisation of hormone peak, nadir and activity times in a cohort of 214 healthy participants. For each parameter, the median (mean) ± SD clock time (hh:mm format) is reported.18-OHF =18-hydroxycortisol, CCS = corticosterone, aTHF = allo-tetrahydrocortisol, THF = tetrahydrocortisol.

	Peak	Nadir		Time
Cortisol	07:38 (08:08) ± 2:55	01:31 (01:03) ± 2:27	**Final wake**	06:59 (07:04) ± 1:16
Cortisone	07:42 (08:33) ± 3:03	01:22 (00:59) ± 2:21	**Sleep attempt**	23:30 (23:28) ± 0:58
18-OHF	07:27 (07:54) ± 2:47	00:42 (00:27) ± 2:18	**Breakfast**	09:30 (09:17) ± 1:30
CCS	08:19 (10:11) ± 5:25	00:12 (00:31) ± 2:47	**Lunch**	12:59 (13:14) ± 1:25
Aldosterone	09:27 (10:33) ± 4:33	00:20 (00:11) ± 3:02	**Dinner**	19:00 (18:59) ± 2:14
aTHF	09:54 (12:17) ± 5:48	01:03 (01:15) ± 2:59		
THF	09:27 (11:09) ± 5:01	01:25 (00:59) ± 2:43		