

2019-09-16

Continuous Free Cortisol Profiles in Healthy Men - Validation of Microdialysis Method

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<http://hdl.handle.net/10026.1/14924>

10.1210/clinem/dgz002

The Journal of Clinical Endocrinology & Metabolism

The Endocrine Society

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1 CONTINUOUS FREE CORTISOL
2 PROFILES IN HEALTHY MEN –
3 VALIDATION OF MICRODIALYSIS
4 METHOD

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21 ABSTRACT:

22 *Context:* In humans, approximately 95% of circulating cortisol is bound to corticosteroid-binding
23 globulin and albumin. It is only the free fraction that is biologically active and can activate signalling
24 pathways via glucocorticoid hormone receptors in cells. Microdialysis is a well-established technique
25 that enables the sampling of molecules in different compartments of the body, including extracellular
26 fluid. This is the first study validating a rapid sampling microdialysis method measuring free cortisol
27 in the subcutaneous and blood compartments of healthy volunteers.

28

29 *Methods:* Healthy non-smoking volunteers (42 men; age 18-24 years; BMI 18-25 kg/m²) received
30 placebo (saline), 250 µg Synacthen or 1 mg dexamethasone with ten minutely sampling to measure
31 total and free cortisol (subcutaneous, intravenous and saliva) for an hour before and 4 hours after
32 administration.

33

34 *Results:* Following stimulation by Synacthen, total serum cortisol and free cortisol in both
35 compartments rose significantly, achieving and maintaining maximum levels between 2 and 3 hours
36 following the stimulus. A decline in cortisol levels was evident after the administration of
37 dexamethasone or placebo, but there was a clear pulsatile activity around lunchtime in the latter group
38 which was prominent in the blood compartment (total and free cortisol). There was good correlation
39 between serum total and free cortisol (SC and intravenous) in the Synacthen and dexamethasone
40 groups with no significant delay (less than 5 minutes) between total and free cortisol.

41

42 *Conclusions:* This seminal study demonstrated the dynamic responses of total blood cortisol and
43 microdialysis derived free cortisol in blood, subcutaneous tissue and saliva in man.

44

45

46 INTRODUCTION:

47 The hypothalamic-pituitary-adrenal (HPA) axis is the key neuroendocrine system responsible
48 for maintaining homeostasis, mediating its effects through glucocorticoid hormones, corticosterone in
49 the rodent and predominantly cortisol in man. Approximately 95% of circulating cortisol in man is
50 inactive as it is bound to plasma proteins such as corticosteroid-binding globulin (CBG; also known
51 as transcortin or SerpinA6¹) and albumin². Only the free fraction of the hormone is biologically
52 active as it activates signalling pathways via binding to glucocorticoid hormone receptors in cells
53 (Figure 1).

54 Microdialysis is a well-established technique that enables the sampling of molecules in
55 different compartments of the body, including extracellular fluid. Microdialysis has originally been
56 developed for monitoring neurotransmitter levels in the brain of rodents and to study the effects of
57 pharmacological interventions³. However, in the early 1990s it was recognised that microdialysis
58 could be applied to the measurement of glucocorticoid hormones in peripheral⁴ and brain tissue⁵ of
59 the rat. This approach opened up the use of in vivo microdialysis for physiological studies with a
60 particular focus on the stress hormone response⁶. Importantly, microdialysis offers the significant
61 advantage of directly measuring the active free component of glucocorticoid hormone, because the
62 size of the membrane pores precludes diffusion of protein-bound glucocorticoid molecules. Using
63 microdialysis in freely behaving rats and mice, Linthorst, Reul and colleagues showed stressor-
64 specific free corticosterone responses in the brain and elucidated the role of corticotropin-releasing
65 factor (CRF), the glucocorticoid receptor (GR) and peripheral CBG in the regulation of free
66 corticosterone levels⁶⁻¹⁰.

67 Recent studies have demonstrated the use of microdialysis to measure free cortisol in healthy
68 controls and in patients undergoing medical and surgical stress (elective coronary artery bypass graft)
69¹¹, in critically ill patients¹² including those in septic shock^{13,14}, and burns patients¹⁵. Free cortisol
70 was measured by equilibrium dialysis^{11,12} or ultrafiltration¹³ in blood, and by microdialysis in the
71 subcutaneous adipose tissue¹⁴, dermis¹⁵ and brain¹⁶. These studies however did not look in detail at
72 dynamic changes in free cortisol levels. Conversely, in rats and mice, high-resolution microdialysis

73 has successfully been used to demonstrate circadian and ultradian free corticosterone rhythms and to
74 capture dynamic free hormone responses to physiological and pharmacological challenges ^{6,9,17}.

75 Within this context, we aimed to translate this approach into high-resolution microdialysis in
76 humans, which will ultimately enable us to study in detail dynamic free cortisol responses during
77 health and disease. We aim to achieve this goal by validating a rapid sampling microdialysis method
78 measuring free cortisol in the subcutaneous and blood compartments of healthy volunteers.

79 Subcutaneous and blood free cortisol levels in dialysates were compared with total serum
80 cortisol levels and free cortisol levels in saliva. The responsiveness of free and total cortisol in the
81 different compartments, and their intricate relationship, were studied by stimulating cortisol release
82 from the adrenal cortex with synthetic ACTH and by suppression of HPA axis activity by
83 dexamethasone. The plasma responses of total cortisol to stimulation with synthetic ACTH or
84 suppression by exogenous dexamethasone are well established and used as diagnostic tests in clinical
85 practice, but there is little information about the effect of these compounds on free cortisol levels in
86 extracellular fluid.

87 MATERIALS AND METHODS:

88 PARTICIPANTS

89 Non-smoking male volunteers (n=42) with normal BMI (18-25 kg/m²), aged 18 to 24 years, were
90 recruited as per local ethical committee regulations (Application reference 08/H0101/16), in
91 accordance with the Helsinki principles. All participants gave informed written consent before their
92 participation in the study. They had no known, past or present, medical conditions, were on no regular
93 pharmacological treatment including oral or parenteral corticosteroid use, and had no known drug
94 allergies.

95

96 MICRODIALYSIS PROCEDURE

97 A. Subcutaneous microdialysis

98 Subcutaneous (SC) microdialysis was performed using a sterile CMA 66 linear microdialysis catheter
99 (Microdialysis AB, Stockholm, Sweden) with polyarylethersulphone membrane, length 30 mm,
100 diameter 0.5 mm, molecular cut-off 20 kDa; polyurethane inlet and outlet tubes 400 and 100 mm,
101 respectively, membrane diameter 0.5 mm. Commercially available sterile physiological perfusion
102 fluid for peripheral tissue use (T1, M Dialysis AB formerly CMA Microdialysis, Stockholm, Sweden;
103 sodium 147mmol/L, potassium 4mmol/L, calcium 2.3mmol/L, chloride 156mmol/L) was used to
104 perfuse the SC catheter.

105

106 B. Intravenous microdialysis

107 Intravenous microdialysis was performed using a sterile MicroEye PME011 (Probe Scientific,
108 Coventry, UK) concentric catheter (polymer membrane, length 15 mm, 0.2 mm diameter, molecular
109 cut-off 9 kDa; PA6228 inlet and outlet tubes 400 mm and 200 mm, respectively). The intravenous
110 catheter was perfused with a sterile mixture of 2 ml of 0.9% saline and 0.5 ml Arixtra (Fondaparinux
111 Sodium, GlaxoSmithKline, Middlesex, UK) for anticoagulation. Prior to use, the outlet tube of each
112 catheter was truncated to 100 mm (to match the length of SC catheter) with a sterile blade and its
113 membrane was dipped in Arixtra to avoid microclots (as per manufacturer's instructions).

114 **C. Sampling**

115 A portable CMA 107 microdialysis pump with a sterile CMA 106 pump syringe (CMA Microdialysis
116 AB, Stockholm, Sweden) at a flow rate of 2 µl/min was used for all experiments. Microdialysates
117 were collected manually in polypropylene vials (300 µl; Royem Scientific Limited, Luton, UK) and
118 stored at -80 until assay.

119

120 **COLLECTION OF BLOOD AND SALIVA SAMPLES**

121 Blood samples were collected as described previously by Henley et al ¹⁸, saliva samples were
122 collected using Salivette® Cortisol synthetic swabs (Sarstedt, Nümbrecht, Germany) and stored at
123 minus 80° C after centrifugation.

124

125 **EXPERIMENTAL DESIGN**

126 Participants arrived at the clinical research unit at least an hour in advance, and intravenous cannulae
127 and microdialysis catheters were inserted and set up at least 45 minutes before the start of the
128 experiment. The participants remained seated in a chair or reclining on a bed throughout the duration
129 of sampling apart from during comfort breaks, when sampling was not interrupted. Standard meals
130 were served at midday for all experiments. Subjects were allowed to carry out work-related activities
131 on their personal computers.

132 An intravenous cannula for blood sampling was inserted in the left ante-cubital fossa, and a SC
133 microdialysis catheter was inserted subcutaneously in the middle part of lateral left upper arm
134 (Experiment 1; Figures 2 and 3) or the lower anterior abdomen (Experiment 2; Figures 2 and 4). For
135 Experiment 2, in addition to the SC microdialysis catheter, a concentric microdialysis catheter was
136 inserted via an 18-G B-Braun intravenous catheter as per manufacturer's recommendation in the right
137 ante-cubital fossa (arm opposite to that used for blood sampling).

138 Both serum and microdialysate samples were collected at ten-minutely intervals (Figures 3 and
139 4). Two sets of experiments were conducted. The differences between the experiments include
140 compartments studied using microdialysis and timing of the dialysates in relation to blood sampling.

141 In the first experiment, the site of the SC catheter was upper arm and dialysate sampling clock-period
142 was 10:00 to 15:00 (n=6). As microdialysate sampling is continuous, the sample reading was
143 considered to represent the midpoint of a given sampling duration e.g. sample timing of 10:05 was for
144 dialysate obtained between 10:00 and 10:10 and so on. In the second experiment, the site of the SC
145 catheter was anterior abdomen, an additional intravenous microdialysis catheter was also inserted and
146 dialysate sampling clock-period was 09:55 to 15:05 (n=8 participants). In both experiments, serum
147 samples were collected at 10:00, 10:10 etc. The dialysate samples in the first experiment correspond
148 to 10:05, 10:15 and so on, and in the second sample timings coincide with that of blood, exactly.
149 Saliva samples were collected every thirty minutes during both sets of experiments starting at 10:00.

150 In both experiments, one of the pharmacological agents [Synacthen 250 µg (Ciba-Geigy,
151 Basel, Switzerland), dexamethasone 1mg (1ml of 3ml saline mixed with 1ml (4mg/ml)
152 dexamethasone, Organon laboratories, Cambridge UK), saline (Sodium chloride 0.9%, Pfizer Ltd,
153 Kent UK)] was administered by 11:02 (Figure 2).

154

155 **ASSAYS**

156 Serum total cortisol concentrations were measured by electrochemiluminescent immunoassay (using a
157 Cobas® e601 immunoassay analyser, Roche Diagnostics, Burgess Hill, UK). Intra-assay and inter-
158 assay precision for serum concentrations of 208, 561 and 1268 nmol/l had coefficients of variation
159 (CV) of 1.3, 1.3, 1.1 % and 1.6, 1.5, 1.6 %, respectively. Dialysate samples were analysed using an
160 ELISA assay for salivary (free) cortisol (IBL, Hamburg, Germany), which was optimised for the
161 measurement of free cortisol in small dialysate volumes (15 µl instead of the recommended 20 µl).
162 The small dialysate volumes allowed a singlicate measurement of each sample. The intra-assay and
163 inter-assay precision for saliva concentrations of 7.45 and 64.58 nmol/l had CVs of 7.3 %, 3.1 % and
164 8.8 %, 6.4 %, respectively. The cross reactivity of the assay with dexamethasone is stated by the
165 manufacturer to be less than 1%. Saliva samples were prepared for analysis using a liquid-liquid
166 extraction procedure with deuterated internal standards (d4-cortisol and d7-cortisone) before isocratic
167 separation UPLC (Waters Acquity, BEH C18) and quantitation by MS-MS (Waters Premier XE).

168 Salivary cortisol and cortisone were analysed with an in-house assay developed by the Department of
169 Clinical Biochemistry, University Hospitals Bristol NHS Foundation Trust, Bristol, United Kingdom
170 using UPLC®-tandem mass spectrometry (Waters Acquity-Premier XE, Waters, Milford, MA 01757
171 USA). The inter-assay CV for cortisol were 17.6 % and 9.9 % for 2 nmol/l and 46 nmol/l respectively,
172 whereas those for cortisone were 6.7 % and 10.4 % for 4 nmol/l and 94 nmol/l respectively. The intra-
173 assay CV for cortisol were 18.3 % and 8.8 % for 2 nmol/l and 46 nmol/l respectively, whereas those
174 for cortisone were 10.2 % and 8.0 % for 4 nmol/l and 94 nmol/l, respectively.

175

176 **STATISTICAL ANALYSES**

177 Analyses were performed with Stata 13 (Stata Corp, College Station, TX, USA).

178 For graphical purposes, Figures 5 and 6 show data as mean \pm SEM. As the data are not normally
179 distributed, median values and ranges have been calculated and are presented in the text. The
180 direction of change was assumed from serum to SC tissue, i.e. the pattern in the former would precede
181 the latter; correlations among serum, SC, and saliva values were estimated using Spearman's rank test
182 for different time lags. In experiment 1, the range of time lag was from 5 to 125 minutes (i.e., values
183 of serum cortisol at time zero were correlated with successive 10-minute values of SC free cortisol,
184 from 5 to 125 minutes: due to the design of the experiment, there was a minimum of five minutes
185 difference between successive serum total and SC free cortisol measurement). In experiment 2, the
186 time lag range was from isotemporal measurements (lag zero) to lag 120 minutes. A limit of either
187 120 (experiment 1) or 125 minutes (experiment 2) was decided as there were no further values after
188 these time points.

189 In order to further examine the relationship between serum total and SC free cortisol, data
190 from the two individual experiments 1 and 2 were combined. Log transformation of this entire data
191 set was carried out and smoothed time-wise mean of these log-transformed values were plotted using
192 the R statistical software¹⁹.

193

195 RESULTS:

196 **Experiment 1: FREE CORTISOL IN THE SC TISSUE COMPARTMENT**

197 Figure 5 shows the expected circadian reduction in both total and SC free cortisol between 10:00 and
198 11:00, before the administration of a pharmacological agent. Following stimulation by Synacthen,
199 there is a marked and rapid rise in cortisol levels in all compartments. Peak levels are achieved
200 between 2 and 3 hours post administration, with a gradual decline in the final hour of sampling. The
201 median serum total cortisol level immediately before Synacthen was 321 nmol/l (range 188-773) and
202 at peak was 973 nmol/l (range 778-1369) between 2 and 3 hours post injection. The median SC tissue
203 free cortisol level pre-Synacthen was 11.5 nmol/l (range 2.3-38.5) and at peak was 67.4 nmol/l (18.7-
204 101.5) between 1 and 3.25 hours post injection.

205 Median serum total cortisol level pre dexamethasone (Figure 5) was 265.0 nmol/l (range
206 169.0-434.0) and nadir level post dexamethasone was 66.0 nmol/l (range 34.0-78.0). For SC tissue
207 free cortisol values, median pre dexamethasone level was 6.0 nmol/l (range 3.2-24.4) and nadir 2.0
208 nmol/l (range 0.9-4.4). Continued decline in cortisol levels is evident after the administration of
209 dexamethasone and placebo, but there was a clear pulsatile activity around lunchtime (midday) in the
210 latter group. This pulse was prominent in serum but less so in SC tissue. Peak level of this pulse in
211 serum cortisol was observed between 30 and 60 minutes after serving lunch and had returned to
212 baseline (pre-lunch levels) latest by 2 hours.

213 Spearman correlation coefficients between serum and SC tissue measurements were
214 calculated for the entire group in each treatment arm (Table 1.1); there was a significant correlation in
215 both the treatment groups but not in the placebo group.

216 In order to address the existence of any delay between serum total and SC free cortisol levels
217 a maximum delay of 125 minutes was considered in keeping with the sampling duration. Spearman
218 correlation coefficient values were computed (Figure 7) at successive 10-minute time delay such that
219 SC would lag behind serum from a minimum of 5 to maximum of 125 minutes. No clear trends in
220 correlation coefficients were found for the placebo and dexamethasone-treated groups; conversely,
221 correlation coefficients for ACTH shows a steady and significant decline with increasing lag times,

222 reflecting the profound increase and subsequent decrease to baseline levels in cortisol over the course
223 of the experiment.

224 Correlations were calculated as in Figure 8 to investigate the relationship between SC tissue
225 free cortisol and that of saliva free cortisol and saliva cortisone. For each of the three interventional
226 groups, the median correlation between saliva cortisone and SC was better (+0.5, +0.83 and +0.9 for
227 the Synacthen, dexamethasone and placebo groups, respectively) than that between saliva free cortisol
228 and SC (+0.21, +0.6 and +0.4, respectively). At baseline, cortisone levels were higher than cortisol
229 levels in saliva and the increase in the latter following Synacthen was more than that in the former.

230

231 **Experiment 2: FREE CORTISOL IN THE SC AND INTRAVENOUS COMPARTMENTS**

232 As in experiment 1, normal circadian fall was seen between 10:00 and 11:00 before the administration
233 of a pharmacological agent (Figure 6). Following stimulation by Synacthen, total serum cortisol as
234 well as free cortisol rose significantly, achieving and maintaining maximum levels between 2 and 3
235 hours following the stimulus, with gradual decline in the final hour of sampling. The median serum
236 total cortisol level immediately before Synacthen was 238.0 nmol/l (range 95.0-375.0) and at peak
237 was 872.0 nmol/l (range 600.0-960.0) between 110 and 150 minutes post injection. The median free
238 cortisol pre-Synacthen in SC tissue level was 6.1 nmol/l (range 0.6-16.75) and in intravenous
239 compartment was 2.7 mmol/l (range 0.6-16.75) with peak of 44.55 nmol/l (28.8-95.7) between 120
240 and 190 minutes post injection, in SC tissue, and 53.6 (range 29.3-101.7) between 120 and 160
241 minutes, in intravenous compartment.

242 Median serum total cortisol level before the administration of dexamethasone was 265.0
243 nmol/l (range 144.0-475.0) and nadir level post dexamethasone was 62.6 nmol/l (range 37.3-122.8).
244 The median pre-dexamethasone level for SC tissue free cortisol was 5.5 nmol/l (range 3.2-11.8) and
245 nadir 0.61 nmol/l (range 0.13-1.76) whereas those for intravenous free cortisol were 5.8 nmol/l (range
246 1.7-9.3) and 0.5 nmol/l (range 0.3-1.3), respectively. As in the first experiment, the difference
247 between placebo and dexamethasone groups was the peak around lunchtime (at midday) in all
248 participants on a background of declining cortisol levels for the duration of sampling (Figure 6). As in

249 experiment 1, this activity was prominent in serum total cortisol levels and less so in SC free cortisol
250 levels, but the intravenous free cortisol also showed the pulsatile rise.

251 Correlation coefficients were calculated for paired combinations of serum total, SC tissue free
252 and intravenous free cortisol for each treatment group (Table 2.1). Similar to Experiment 1, whereas
253 the correlations for the placebo group was not statistically significant, strong and significant
254 correlations between cortisol in the different compartments were found in the stimulation and
255 suppression groups.

256 In order to investigate whether there exists a possible delay between serum and SC, serum and
257 intravenous free levels, and between SC and intravenous free levels, correlations were computed for
258 time-lag range of 0 to 120 minutes (Figure 9) at successive 10-minute time delay such that SC would
259 lag behind serum from a minimum of 10 to maximum of 120 minutes. There was no significant trend
260 in placebo and dexamethasone groups, with the best positive correlation at time zero. For Synacthen
261 group, however there was a trend such that the median correlation value at 35 minutes (+0.52) was
262 nearly equally inverse of that at 120 minutes (-0.58). This suggests that 120 minutes following a pulse
263 of cortisol, SC value would be expected to decline further supporting the previous data.

264 Correlation coefficients were calculated to examine the relationship between SC tissue free
265 cortisol and saliva free cortisol and saliva cortisone (Figure 10). The strongest median correlation was
266 found for the dexamethasone group (0.88), and it was true for both saliva fractions. The relationships
267 for the remaining two treatment groups were weaker (0.3-0.5) for both saliva fractions. At baseline,
268 cortisone levels were higher than cortisol levels in saliva and the increase in the latter following
269 Synacthen was more than that in the former fraction.

270 In order to further examine the relationship between serum total and SC free cortisol, data
271 from the two experiments were combined. There was a strong evidence of effect of treatment in each
272 of the three groups (Figure 11). In the dexamethasone group, the mean values from both the
273 experiments continue to decline throughout the experiment like in the placebo group, apart from the
274 mealtime peak in the latter group as previously mentioned. The mean peak values from the two
275 experiments following Synacthen overlap suggesting maximum stimulation at two hours (regardless

276 of baseline levels). The mean nadir values from the two experiments remain separated in the
277 dexamethasone group, however in the placebo groups there is a hint of an upward deflection in the
278 second set of experiments.

279 Cross-correlation plots of log transformed serum total and SC free cortisol for each individual
280 in each of the treatment arms of the two studies are shown in Figure 12. This data confirmed previous
281 analysis by demonstrating significant positive cross-correlation between serum and SC values across
282 all participants in the Synacthen and dexamethasone groups. However, there was variability in the
283 placebo group with poorer cross-correlation across the group, confirmed by the non-significant
284 Spearman correlation coefficients calculated previously.

285

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288

289 DISCUSSION:

290 To our knowledge, this is the first study demonstrating the relationship between total cortisol
291 in serum and free cortisol in three body compartments. We compared the dynamic responses of total
292 blood cortisol and microdialysis derived free cortisol in blood, subcutaneous tissue and saliva in man.
293 The only studies to have compared the dynamic responses of both blood and tissue glucocorticoid
294 levels have been performed in the rat. A landmark series of studies of free corticosterone rhythms in
295 different body compartments were performed using stress paradigm in which animals are forced to
296 swim in water at 25°C. In these studies peak free hormone levels in the intravascular compartment,
297 SC tissue ⁹ and in the brain (hippocampus) ^{6,17}, were found 20 minutes later than the peak of total
298 hormone in the plasma.

299 Rather than using a stress manoeuvre, we used the Synacthen test, first described by Wood to
300 ascertain adrenocortical reserve ²⁰ and which can be performed at any time of the day ²¹. It has been
301 shown to correlate well with responses to major surgical stress ^{11,22}. There is a large literature relating
302 to serum total cortisol levels at time 0 and 30 (and/or 60) minutes after Synacthen, but little is known
303 about the effect on the pattern of free cortisol levels in a tissue, other than saliva ²³.

304 There was good correlation between serum total and free cortisol (SC and intravenous) in the
305 Synacthen and dexamethasone groups, with no discrimination between compartments. Forced swim
306 stress in rodents showed a twenty-minute delay in free corticosterone rise in the hippocampus, blood
307 and subcutaneous tissue, compared to total corticosterone in blood ^{6,9} most likely as a consequence of
308 a stressor-specific rise in circulating CBG from the liver ⁹. This was not the case in our studies
309 although delay of less than 5 minutes, if present, could not be ruled out due to the sampling frequency
310 used in this study. The relationship between serum total and free cortisol (SC and intravenous) is not
311 as strong in the placebo group, which is expected in a short sampling duration due to individually
312 distinct secretory pulse patterns. With stimulation and suppression, the axis was entrained resulting in
313 better correlation, whereas in the placebo group inter-individual variation in pulses is likely to have
314 affected the correlation values.

315 There was evidence of some secretory activity around noon in the placebo group – the only
316 difference between placebo and dexamethasone groups. The clearly visible peak in serum was not
317 discernible in the SC tissue in the majority of subjects, but was visible in the intravenous free fraction.
318 This was especially true when the serum total cortisol peak was 400 nmol/l or more and, when serum
319 total cortisol was at or above this level a change in SC levels was more noticeable. This may relate to
320 the fact that this is the level of plasma cortisol at which CBG becomes saturated and any further
321 change in cortisol results in a disproportionately large increase in free cortisol ²⁴. Changes in the level
322 of CBG or its affinity for cortisol via neutrophil elastase or changes in temperature can also alter free
323 cortisol fractions ²⁵. Change in albumin levels affecting free cortisol levels is relatively less strong ¹¹.
324 In our cohort of normal individuals, there is no reason to expect abnormalities of albumin or CBG
325 levels or that there would be changes in body temperature known to markedly alter affinity of CBG
326 for cortisol ²⁴. Lunch was served to all individuals in the study groups at noon.

327 The Synacthen studies produced a prompt and marked rise in plasma and free cortisol
328 followed by decline in levels 120-125 minutes later in both the SC and intravenous compartments.
329 The pharmacodynamics of Synacthen (1-24) are somewhat different to endogenous ACTH (1-39),
330 with high levels evident up to 60 min following an intravenous injection of high dose Synacthen ²⁶.
331 Serum total cortisol levels post 250 µg of Synacthen continue to increase at least until 75 minutes ²⁶.
332 Therefore, an endogenous cortisol pulse (of a shorter duration and consequent drop to lower levels)
333 ^{27,28}, with no further hormone secretion would lead to drop in free cortisol levels in the SC tissue. This
334 phenomenon may also be related to the saturation kinetics of CBG. Total and free cortisol remain in
335 equilibrium in the serum, and free unbound cortisol will readily diffuse into tissues resulting in a rise
336 in tissue levels soon after a pulse. With no further adrenal secretion of cortisol, if plasma cortisol
337 levels dropped, CBG would no longer be saturated and there could be a concentration gradient of free
338 cortisol from subcutaneous tissue to the blood compartment. The half-life of free cortisol in
339 subcutaneous tissue is not known but could also contribute to this relationship.

340 Exclusion of female participants, to avoid problems associated with menstrual cycle
341 dependent changes in the levels of oestrogen and progesterone and their resulting effects on levels of

342 CBG and competition for binding to CBG, respectively, is a limitation of this study. We have
343 attempted to exclude methodological factors that could influence our data. Both the free cortisol
344 fractions for each individual were assayed in the same batch by the same method hence to minimise
345 inaccuracies related to inter-assay variation. The membrane length of the SC was twice that of
346 intravenous microdialysis probe, which could potentially result in differential recovery rates. In
347 reality, the difference in recovery of cortisol from the two probes in *in-vitro* experiments was minimal
348 (about 10%), not requiring an adjustment-factor for membrane length. To avoid discrepancies related
349 to post prandial release of cortisol all of our participants received a standard lunch (commercially
350 available ‘healthy’ range sandwich, orange juice and piece of fruit), providing consistency of food
351 intake around the midday/meal related peak ^{29,30}.

352 The relationship between saliva cortisol and cortisone with SC free cortisol was not the
353 primary focus of this study. Infrequent (half hourly) sampling and differential (non-identical) timing
354 in relation to dialysates in the two experiments meant that pooling the data was of no benefit (unlike
355 the pooling of serum total and SC free cortisol). Cortisone appeared to be a better correlate of SC free
356 cortisol as described previously ²³.

357 In this study we have sought to ascertain how levels of free cortisol in both the blood and
358 subcutaneous compartments of the body correlate with levels of total cortisol in the blood using both a
359 stimulus with Synacthen and inhibition by dexamethasone. In both situations we found good
360 correlation with total cortisol. We have found no significant delay between total levels in the blood
361 and free levels in the blood and subcutaneous tissue although it was not possible to detect a delay of
362 less than 5 minutes. Despite these good correlations, smaller endogenous pulses appeared to not be
363 readily detected in the SC tissue. Investigating the relationship between serum total and SC free
364 cortisol during undisturbed endogenous secretory activity will need further investigation. This study
365 should now pave the way for investigating the levels of free cortisol certainly in subcutaneous and
366 possibly other tissues and thereby truly understanding the role of cortisol in disease pathophysiology,
367 particularly in conjunction with the previously reported ambulatory sampling device ³¹.

368

370 ACKNOWLEDGEMENTS: This study has been funded by OPTIMI (EU-Grant FP7 248544). We
371 thanks all healthy volunteer participants, and Dr W Woltersdorf (formerly of affiliation⁶) for his
372 assistance with the use of chemical pathology laboratory.

373

374 REFERENCES:

- 375 1. MILLS IH. TRANSPORT AND METABOLISM OF STEROIDS. *Br Med Bull.*
376 1962;18(2):127-133. doi:10.1093/oxfordjournals.bmb.a069953
- 377 2. Murray D. Cortisol Binding To Plasma Proteins In Man In Health, Stress And At Death. *J*
378 *Endocrinol.* 1967;39(4):571-591. doi:<https://doi.org/10.1677/joe.0.0390571>
- 379 3. Harp T. ZT. What did we learn from microdialysis? In: *Handbook of Microdialysis: Methods,*
380 *Application and Clinical Aspects.* ; 2007.
- 381 4. Jasper M, Engeland W. Synchronous ultradian rhythms in adrenocortical secretion detected by
382 microdialysis in awake rats. *Am J Physiol.* 1991. doi:10.1152/ajpregu.1991.261.5.R1257
- 383 5. Linthorst ACE, Flachskamm C, Holsboer F, Reul JMHM. Local administration of recombinant
384 human interleukin-1 β in the rat hippocampus increases serotonergic neurotransmission,
385 hypothalamic-pituitary-adrenocortical axis activity, and body temperature. *Endocrinology.*
386 1994. doi:10.1210/endo.135.2.7518383
- 387 6. Droste SK, De Groote L, Atkinson HC, Lightman SL, Reul JMHM, Linthorst ACE.
388 Corticosterone levels in the brain show a distinct ultradian rhythm but a delayed response to
389 forced swim stress. *Endocrinology.* 2008. doi:10.1210/en.2008-0103
- 390 7. Linthorst AC, Flachskamm C, Müller-Preuss P, Holsboer F, Reul JM. Effect of bacterial
391 endotoxin and interleukin-1 beta on hippocampal serotonergic neurotransmission, behavioral
392 activity, and free corticosterone levels: an in vivo microdialysis study. *J Neurosci.* 1995.
- 393 8. Linthorst AC, Flachskamm C, Hopkins SJ, et al. Long-term intracerebroventricular infusion of
394 corticotropin-releasing hormone alters neuroendocrine, neurochemical, autonomic, behavioral,
395 and cytokine responses to a systemic inflammatory challenge. *J Neurosci.* 1997.
- 396 9. Qian X, Droste SK, Gutiérrez-Mecinas M, et al. A rapid release of corticosteroid-binding

- 397 globulin from the liver restrains the glucocorticoid hormone response to acute stress.
398 *Endocrinology*. 2011. doi:10.1210/en.2011-1008
- 399 10. Linthorst ACE, Peñalva RG, Flachskamm C, Holsboer F, Reul JMHM. Forced swim stress
400 activates rat hippocampal serotonergic neurotransmission involving a corticotropin-releasing
401 hormone receptor-dependent mechanism. *Eur J Neurosci*. 2002. doi:10.1046/j.1460-
402 9568.2002.02400.x
- 403 11. Christ-Crain M, Jutla S, Widmer I, et al. Measurement of serum free cortisol shows discordant
404 responsivity to stress and dynamic evaluation. *J Clin Endocrinol Metab*. 2007;92(5):1729-
405 1735. doi:10.1210/jc.2006-2361
- 406 12. Hamrahian AH, Oseni TS, Arafah BM. Measurements of Serum Free Cortisol in Critically Ill
407 Patients. *N Engl J Med*. 2004;350(16):1629-1638. doi:10.1056/NEJMoa020266
- 408 13. Ho JT, Al-Musalhi H, Chapman MJ, et al. Septic shock and sepsis: A comparison of total and
409 free plasma cortisol levels. *J Clin Endocrinol Metab*. 2006;91(1):105-114.
410 doi:10.1210/jc.2005-0265
- 411 14. Vassiliadi DA, Ilias I, Tzanela M, et al. Interstitial cortisol obtained by microdialysis in
412 mechanically ventilated septic patients: Correlations with total and free serum cortisol. *J Crit*
413 *Care*. 2013. doi:10.1016/j.jcrc.2012.07.008
- 414 15. Cohen J, Deans R, Dalley A, Lipman J, Roberts MS, Venkatesh B. Measurement of tissue
415 cortisol levels in patients with severe burns: a preliminary investigation. *Crit Care*. 2009.
416 doi:10.1186/cc8184
- 417 16. Llompарт-Pou JA, Pérez G, Raurich JM, et al. Loss of cortisol circadian rhythm in patients
418 with traumatic brain injury: A microdialysis evaluation. *Neurocrit Care*. 2010.
419 doi:10.1007/s12028-010-9399-1
- 420 17. Qian X, Droste SK, Lightman SL, Reul JMHM, Linthorst ACE. Circadian and ultradian
421 rhythms of free glucocorticoid hormone are highly synchronized between the blood, the
422 subcutaneous tissue, and the brain. *Endocrinology*. 2012. doi:10.1210/en.2012-1484
- 423 18. Henley DE, Leendertz JA, Russell GM, et al. Development of an automated blood sampling

- 424 system for use in humans. *J Med Eng Technol.* 2009. doi:10.1080/03091900802185970
- 425 19. R Development Core Team . R: A Language and Environment for Statistical Computing. *R*
426 *Found Stat Comput.* 2011. doi:10.1007/978-3-540-74686-7
- 427 20. Wood JB, James VHT, Frankland AW, Landon J. A RAPID TEST OF ADRENOCORTICAL
428 FUNCTION. *Lancet.* 1965. doi:10.1016/S0140-6736(65)91526-6
- 429 21. McGill PE, Greig WR, Browning MC, Boyle JA. Plasma cortisol response to synacthen (beta-
430 1-24 Ciba) at different times of the day in patients with rheumatic diseases. *Ann Rheum Dis.*
431 1967;26(2):123-126. doi:10.1136/ard.26.2.123
- 432 22. Widmer IE, Puder JJ, König C, et al. Cortisol response in relation to the severity of stress and
433 illness. *J Clin Endocrinol Metab.* 2005;90(8):4579-4586. doi:10.1210/jc.2005-0354
- 434 23. Perogamvros I, Keevil BG, Ray DW, Trainer PJ. Salivary cortisone is a potential biomarker
435 for serum free cortisol. *J Clin Endocrinol Metab.* 2010;95(11):4951-4958.
436 doi:10.1210/jc.2010-1215
- 437 24. Cameron A, Henley D, Carrell R, Zhou A, Clarke A, Lightman S. Temperature-responsive
438 release of cortisol from its binding globulin: A protein thermocouple. *J Clin Endocrinol*
439 *Metab.* 2010. doi:10.1210/jc.2010-0942
- 440 25. Henley DE, Lightman SL. New insights into corticosteroid-binding globulin and
441 glucocorticoid delivery. *Neuroscience.* 2011;180:1-8. doi:10.1016/j.neuroscience.2011.02.053
- 442 26. Alía P, Villabona C, Giménez O, Sospedra E, Soler J, Navarro MA. Profile, mean residence
443 time of ACTH and cortisol responses after low and standard ACTH tests in healthy volunteers.
444 *Clin Endocrinol (Oxf).* 2006;65(3):346-351. doi:10.1111/j.1365-2265.2006.02602.x
- 445 27. Veldhuis JD, Iranmanesh A, Lizarralde G, Johnson ML. Amplitude modulation of a burstlike
446 mode of cortisol secretion subserves the circadian glucocorticoid rhythm. *Am J Physiol.* 1989.
447 doi:10.1152/ajpendo.1989.257.1.E6
- 448 28. Veldhuis JD, Iranmanesh A, Johnson ML, Lizarralde G. Amplitude, but not frequency,
449 modulation of adrenocorticotropin secretory bursts gives rise to the nyctohemeral rhythm of
450 the corticotropic axis in man. *J Clin Endocrinol Metab.* 1990. doi:10.1210/jcem-71-2-452

- 451 29. Stimson RH, Mohd-Shukri NA, Bolton JL, Andrew R, Reynolds RM, Walker BR. The
452 postprandial rise in plasma cortisol in men is mediated by macronutrient-specific stimulation
453 of adrenal and extra-adrenal cortisol production. *J Clin Endocrinol Metab.* 2014;99(1):160-
454 168. doi:10.1210/jc.2013-2307
- 455 30. Follenius M, Brandenberger G, Hietter B, Siméoni M, Reinhardt B. Diurnal cortisol peaks and
456 their relationships to meals. *J Clin Endocrinol Metab.* 1982. doi:10.1210/jcem-55-4-757
- 457 31. Bhake RC, Leendertz JA, Linthorst ACE, Lightman SL. Automated 24-hours sampling of
458 subcutaneous tissue free cortisol in humans. *J Med Eng Technol.* 2013.
459 doi:10.3109/03091902.2013.773096
- 460

TABLES AND FIGURES (Order of appearance within the text)

Figure 1. HYPOTHALAMIC-PITUITARY-ADRENAL-TISSUE AXIS. CRH from the hypothalamus stimulates ACTH release from the anterior pituitary resulting in cortisol production from the adrenal glands. Free cortisol thus released in the vascular system circulates as protein-bound ($\approx 95\%$) and free ($\approx 5\%$) fractions. Free hormone enters tissues, then into each cell where it binds its receptors (only glucocorticoid receptor GR represented in this figure). Hormone-receptor complex translocates from the cytoplasm to the nucleus, attaches to the glucocorticoid response element on the DNA triggering a cascade of events leading to hormone actions. Liver and adipose tissue predominantly convert circulating cortisone to active cortisol mediated by 11β HSD-1 while 11β HSD-2 in the saliva & kidney converts cortisol into inactive cortisone.

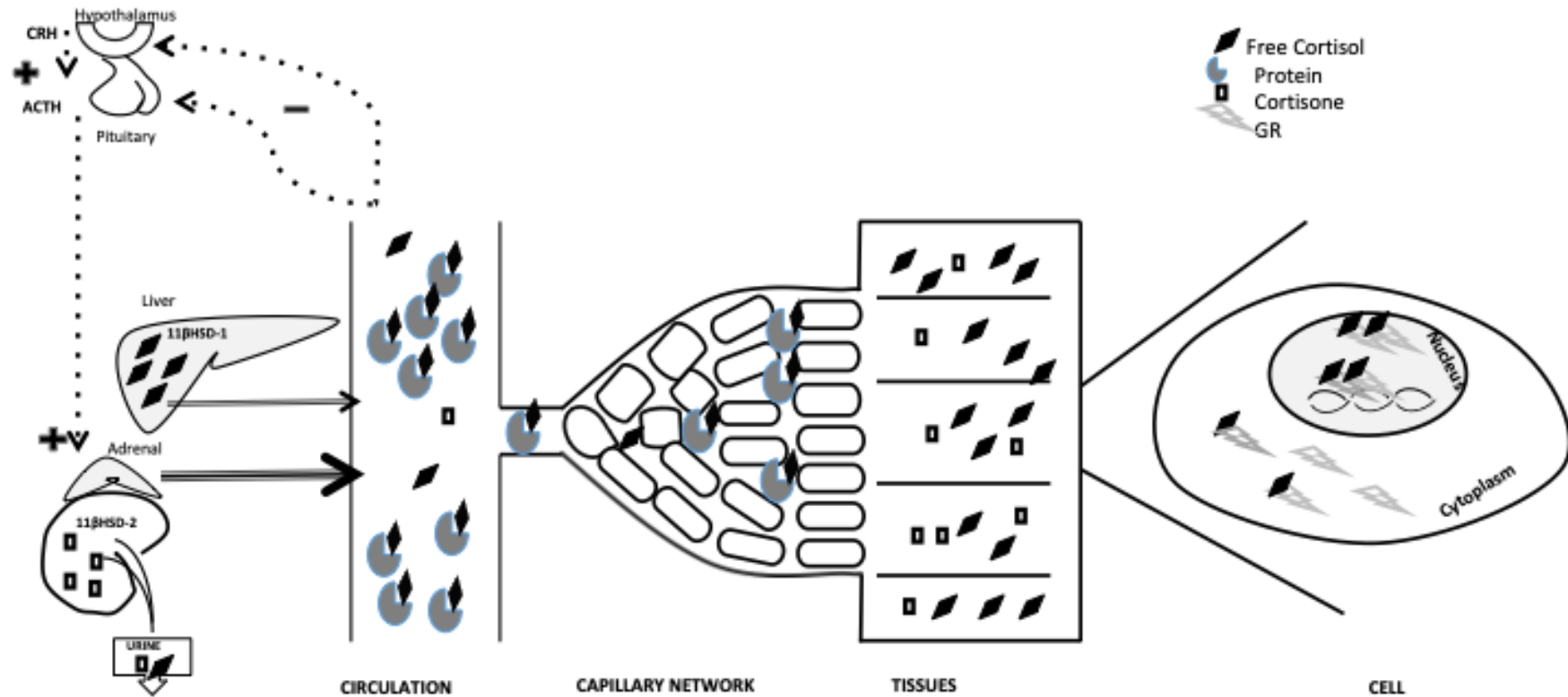


Figure 2. DESIGN OF EXPERIMENTS 1 & 2. All catheter insertions were completed by 09:15. Sampling period of experiment 1 was 10:00 to 15:00 and 09:55 to 15:05 of experiment 2. Pharmacological agent, including saline/placebo, was injected by 11:02. Lunch was served at 12:00.

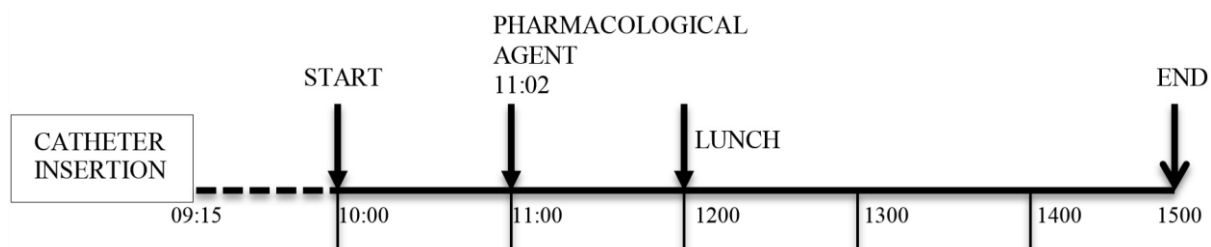


Figure 3. PROTOCOL FOR EXPERIMENT 1. Ten minutely sampling was done from 10:00 to 15:00 manually for SC free cortisol and via automated sampling for blood. Pharmacological agent including saline/placebo was injected by 11:02. Lunch was served at 12:00.

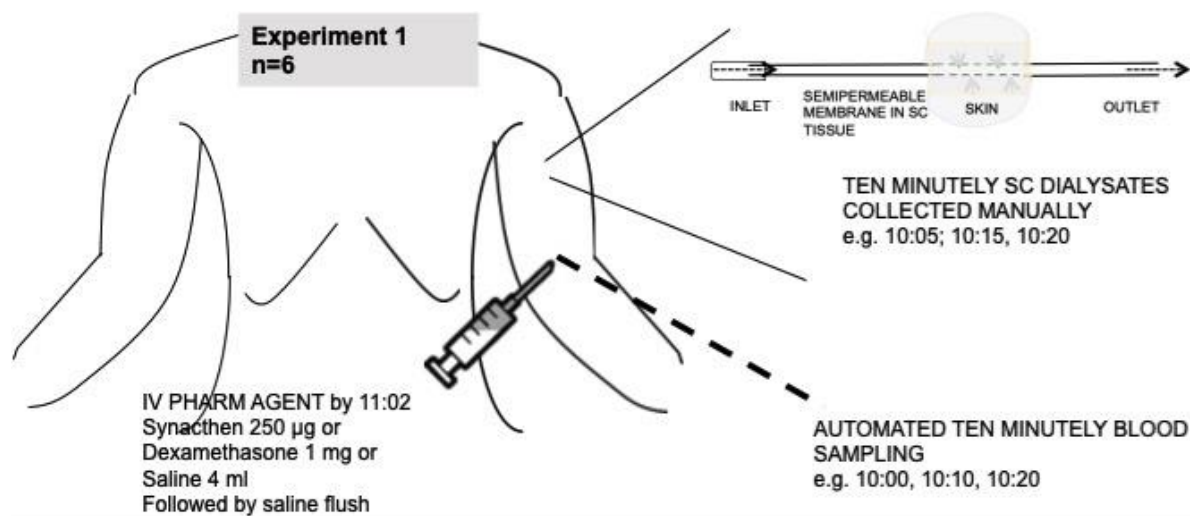


Figure 4. PROTOCOL FOR EXPERIMENT 2. Ten minutely sampling was done from 09:55 to 15:05 manually for SC and IV free cortisol. Automated sampling of blood was done from 10:00 to 15:00. Pharmacological agent including saline/placebo was injected by 11:02. Lunch was served at 12:00.

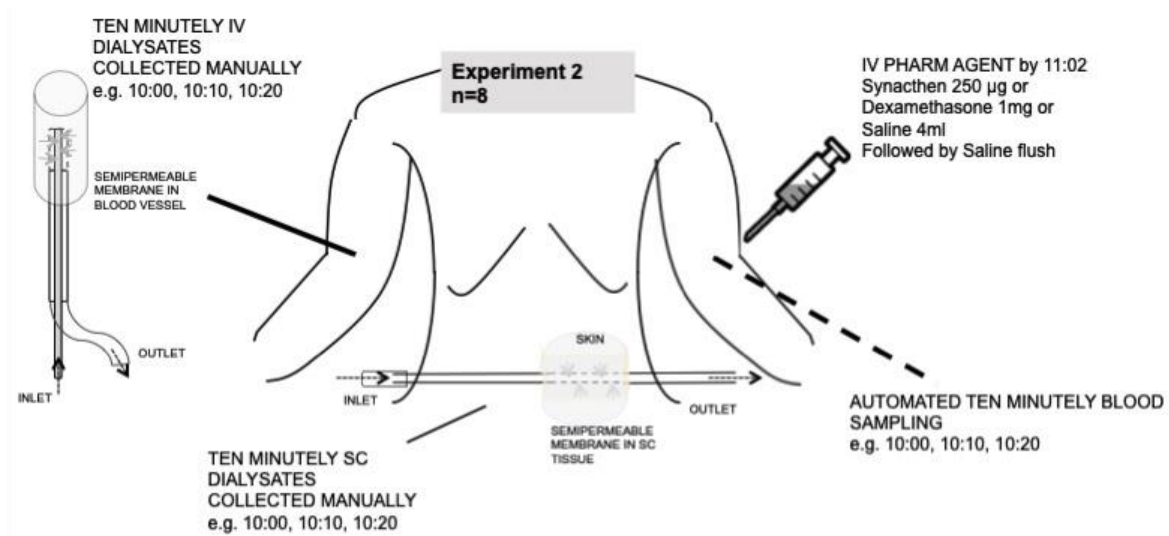


Figure 5. GROUP (a,c,e – mean & SEM) and TYPICAL PROFILES WITHIN EACH GROUP (b,d,f) OF CORTISOL IN SERUM (Total), SC TISSUE (Free Fraction) & SALIVA (Free Fraction And Cortisone). Saline/placebo (a,b); 250 µg Synacthen (c,d) or 1mg dexamethasone (e,f) were administered by 11:02 hours. Sampling commenced at 10:00 with ten-minutely samples collected until 15:00. Saliva samples were collected every half hour starting at 10:00. Lunch was served at 12:00.

Figure 5. Results Of Experiment 1

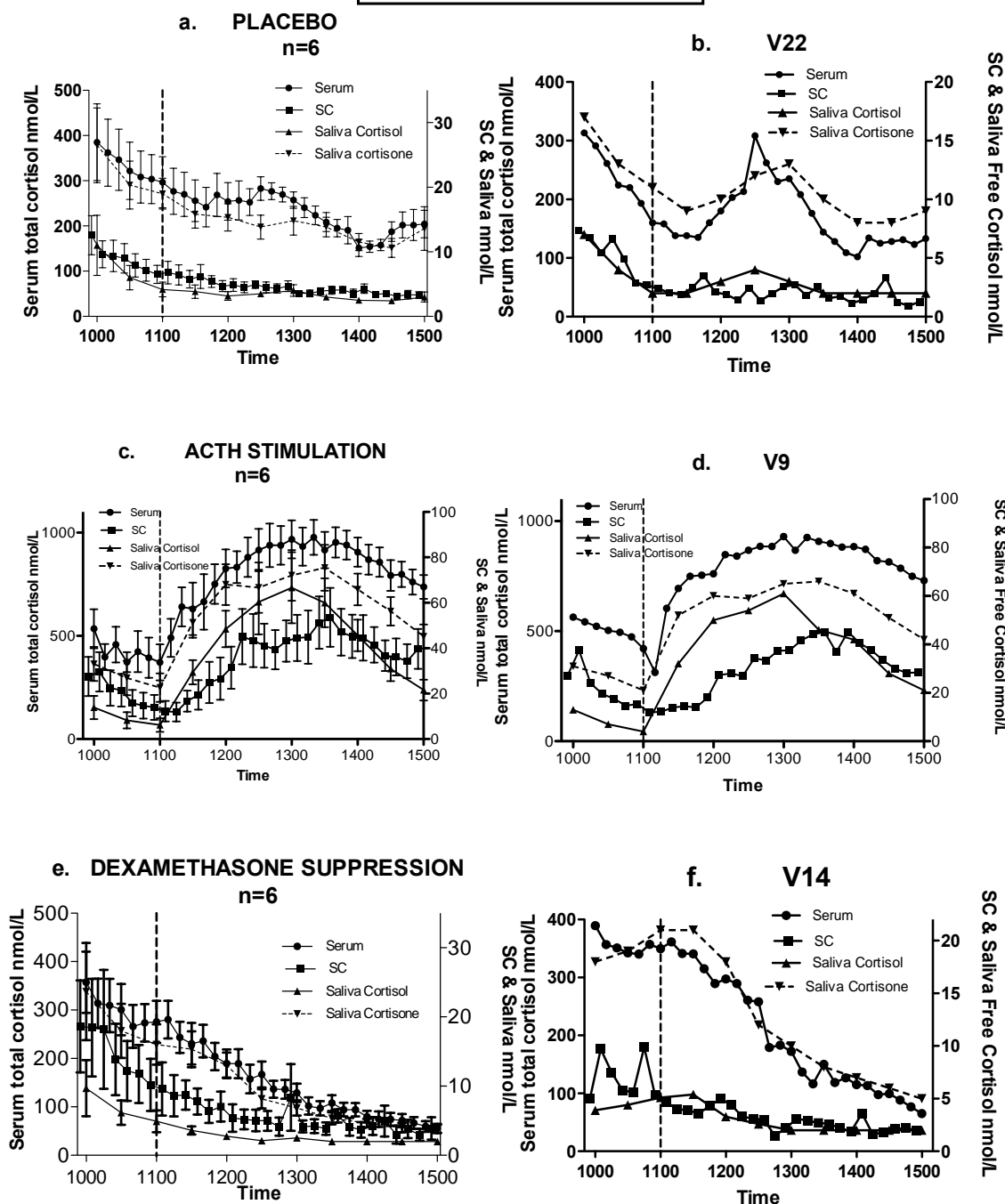


Figure 6. GROUP (a,c,e – mean & SEM) and TYPICAL PROFILES WITHIN EACH GROUP (b,d,f) OF CORTISOL IN SERUM (Total) and in SUBCUTANEOUS & INTRAVENOUS COMPARTMENTS (Free Fraction). Saline (a,b); 250 µg Synacthen (c,d) or 1mg Dexamethasone (e,f) were administered by 11:02 hours. Sampling commenced at 10:00 for serum, and at 09:55 for SC & IV with ten minutely samples being collected up to 15:00. Lunch was served at 12:00.

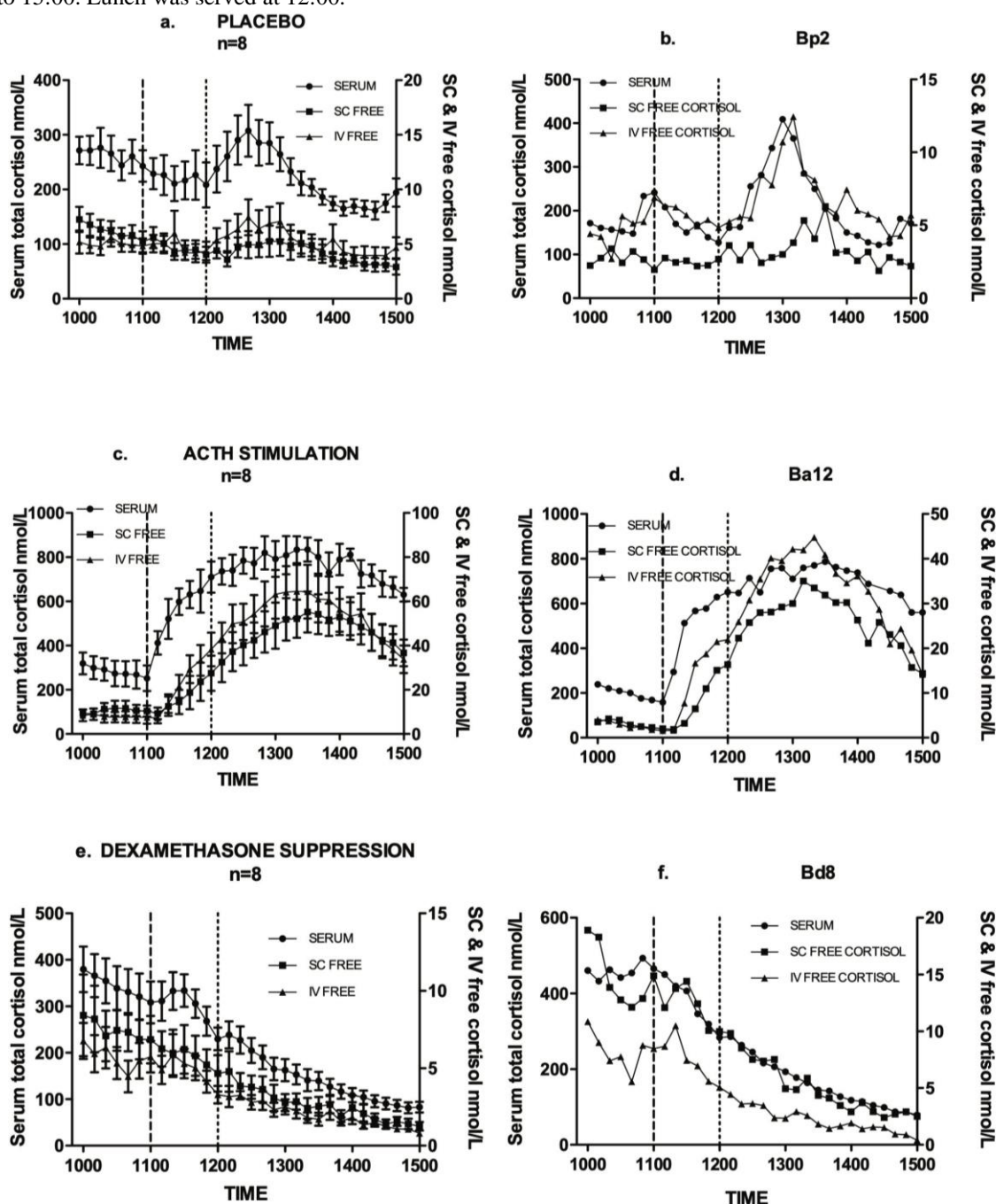


Table 1 CORRELATION BETWEEN SERUM AND SC CORTISOL AT TIME LAG 5 min (minimum time difference in this study).

TABLE 1.1 CORRELATION BETWEEN SERUM AND SC CORTISOL AT TIME LAG 5 min		
TREATMENT (n=6)	SPEARMAN CORRELATION COEFFICIENT	p VALUE
ACTH	0.75	<0.0001
DEXAMETHASONE	0.68	<0.001
PLACEBO	0.36	0.102

Figure 7. SPEARMAN CORRELATION COEFFICIENT VALUES (Y AXIS) BETWEEN SERUM AND SC AT SUCCESSIVE 5-MINUTE TIME DELAY FROM 5 TO 125 MINUTES (X-AXIS). Squares indicate median values, vertical bars the interquartile range and dots are individual participant values. There is no significant trend in placebo and dexamethasone groups.

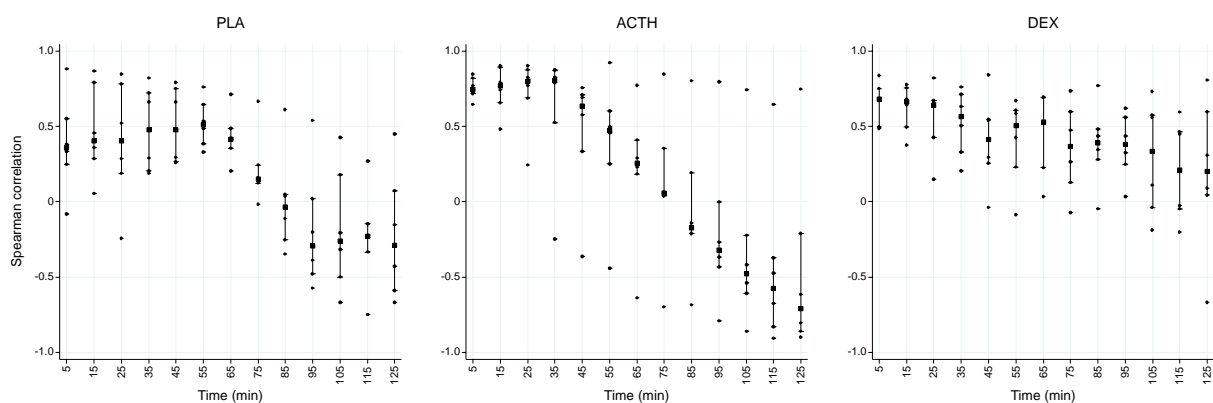


Figure 8. SPEARMAN CORRELATION BETWEEN SC AND EITHER SALIVA FREE CORTISOL (f) OR CORTISONE (e) FOR EACH OF THE TREATMENT GROUPS (SynACTHen stimulation, DEXamethasone suppression or PLAceto) at time lag of 5 minutes. Squares indicate median values, vertical bars the interquartile range and dots denote individual participant values.

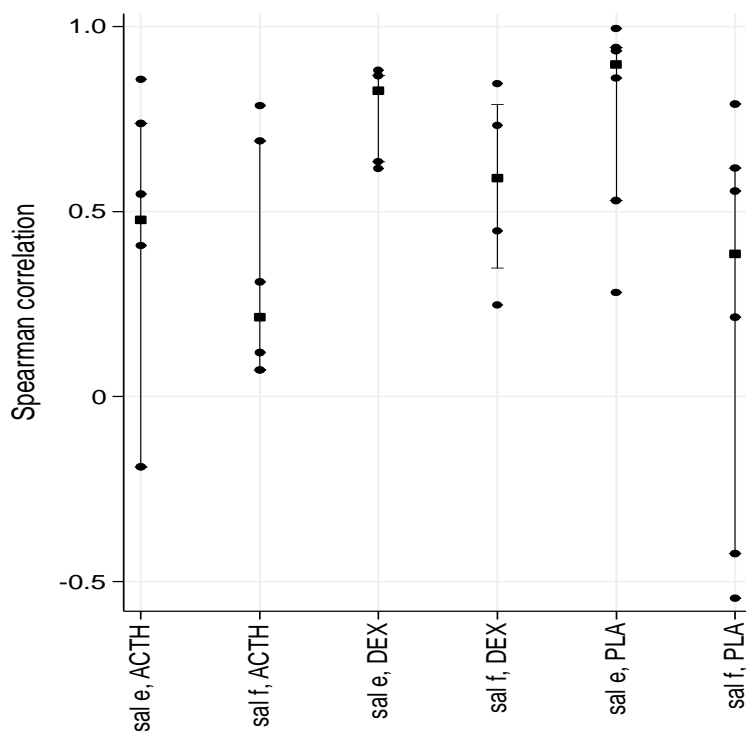


Table 2 CORRELATION COEFFICIENTS AT TIME LAG 0.

TABLE 2.1 CORRELATION COEFFICIENTS AT TIME LAG 0			
TREATMENT	SERUM & SC Free	SERUM & IV Free	SC Free & IV Free
ACTH	0.68 (p=0.001)	0.76 (p<0.001)	0.82 (p<0.001)
DEXAMETHASONE	0.82 (p<0.001)	0.86 (p<0.001)	0.82 (p<0.001)
PLACEBO	0.21 (p=0.322)	0.55 (p=0.008)	0.25 (p=0.309)

Figure 9. SPEARMAN CORRELATION COEFFICIENT VALUES (Y AXIS) BETWEEN SERUM AND SC (bottom row); SERUM AND INTRAVENOUS (IV) free (middle row) AND, SC AND IV FREE (top row) CORTISOL AT SUCCESSIVE 10-MINUTE TIME DELAY FROM 0 TO 120 MINUTES (X-axis). SynACTHen group results are in the left column, DEXamethasone in the middle column, and PLACebo in the right column. Squares indicate median values, vertical bars the interquartile range and dots are individual participant values. There is no significant trend in placebo and dexamethasone groups. For ACTH group, there is a trend such that the median correlation value at time 0 is nearly equally inverse at 120minutes.

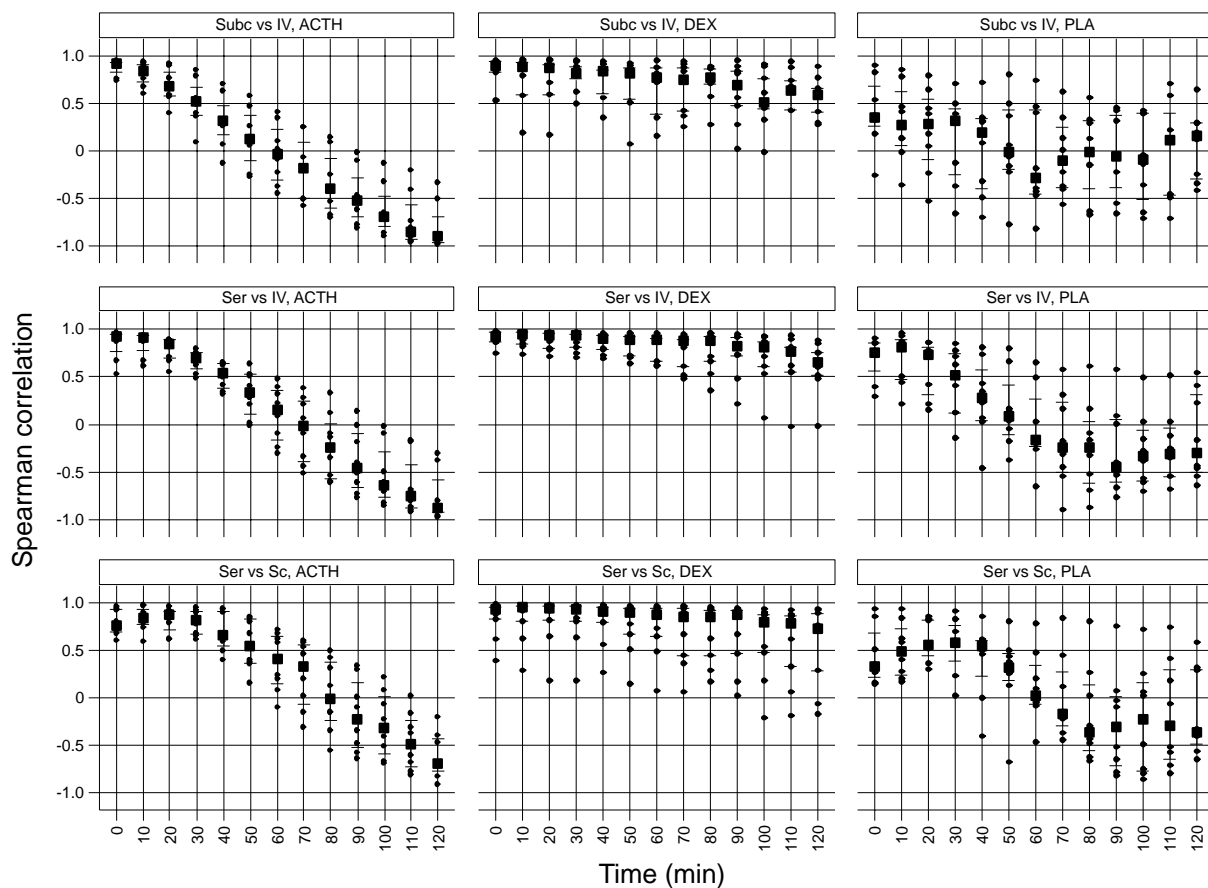


Figure 10. SPEARMAN CORRELATION (Y-axis) BETWEEN SC AND EITHER SALIVA FREE CORTISOL (f) OR CORTISONE (e) PER TREATMENT GROUPS (ACTH stimulation, DEXamethasone suppression or PLAceto) at isotemporal lag time. Squares indicate median values, vertical bars the interquartile range and dots denote individual participant values.

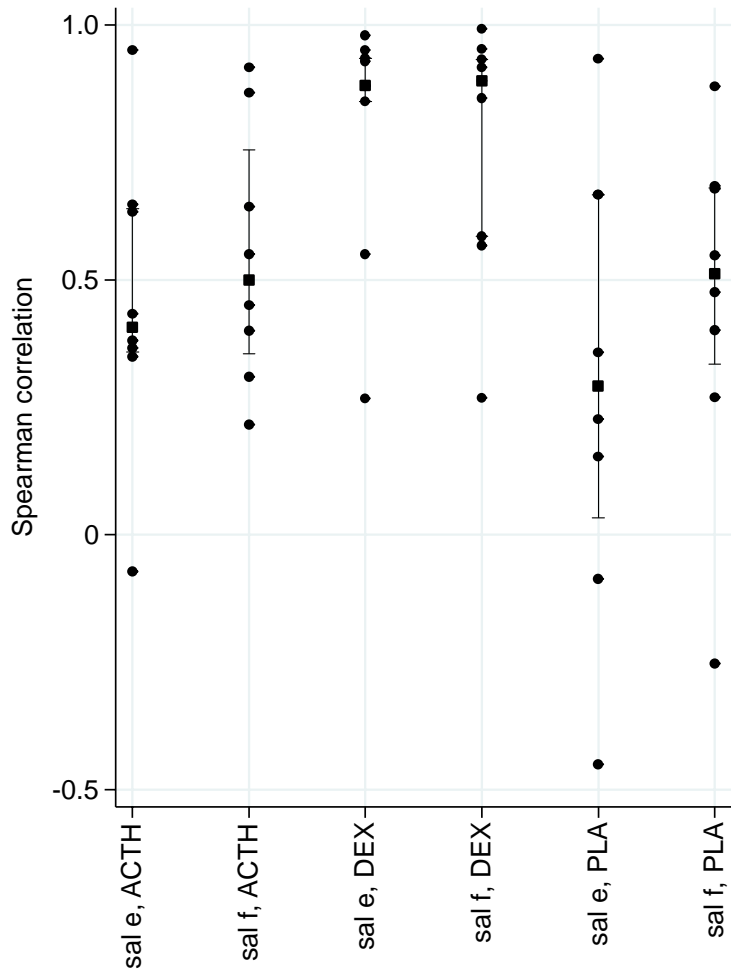


Figure 11. LOG TRANSFORMED MEAN VALUES OF SERUM TOTAL (UPPER PANEL) AND SC FREE CORTISOL (LOWER PANEL) FROM THE TWO PHARMACOLOGICAL MANIPULATION EXPERIMENTS. Bold line represents smoothed mean of all values and shaded area represents 95% confidence intervals. Dotted vertical lines indicate time of pharmacological intervention e.g. Synacthen, Dexamethasone or placebo. X-axis displays clock time.

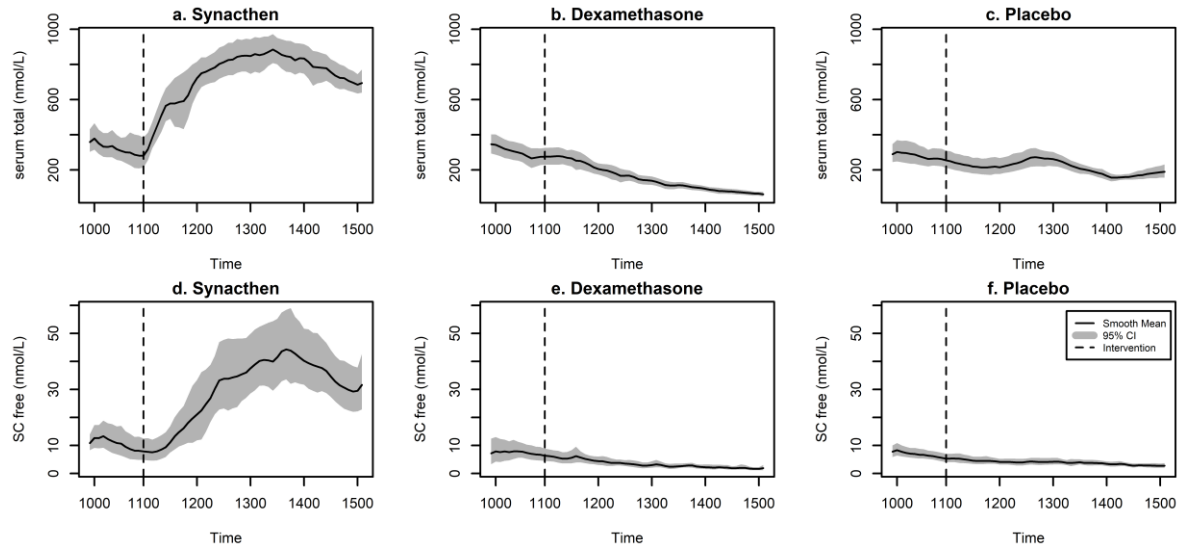


Figure 12. CROSS-CORRELATION PLOTS OF LOG TRANSFORMED SERUM TOTAL AND SC FREE CORTISOL for representative individuals from the two experiments described in the text. ACF (autocorrelation factor) is on Y-axis, time lag on X-axis (lag 1=10min). Plots on the left are three typical individuals per treatment group from experiment one and those on the right from experiment two.. The top row=Synacthen group, middle row=dexamethasone group and the bottom row=the placebo group. Dotted lines indicate confidence intervals. Where vertical lines exceed dotted line (top two rows), there is a significant cross correlation.

