Supplementary material for

Diurnal Production of Cortisol and Prediction of Treatment Response in Rheumatoid Arthritis: a 6-month, Real-life Prospective Cohort Study

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Supplementary Methods

Cohort description

Baseline disease activity was moderate and high in 11 and 17 patients, respectively, with a mean DAS28-ESR of 5.0 ± 1.2 (ranging from 3.7 to 7.25) [Tender joints: mean number: 11; range between 3-24; swollen joints: mean number: 5, range between 0-20; Vas-Patient: mean value: 6.3 range between 2-9].

Eighteen patients were started on csDMARDs alone +/- glucocorticoids, 6 patients received csDMARDs combined with bDMARD or tsDMARDs, whereas 4 patients received monotherapy with bDMARD or tsDMARDs. Twenty three out of the 28 patients were started on glucocorticoid treatment with a mean starting dose of 10 mg of prednisone or equivalent and a tapering dose of 2.5 mg prednisone or equivalent /3-4 weeks, aiming at discontinuation as soon as possible, as recommended [25].

At 6 months, 15/28 (53.6%) of the patients were on treatment with csDMARDs alone, or combined with bDMARD or tsDMARDs (7/28, 25%), whereas 6/28 (21.4%) of patients were receiving monotherapy with bDMARD or tsDMARDs. Of the 23 patients who started glucocorticoids at baseline, 8 and 10 had stopped at 3 and 6 months, respectively. The mean cumulative dose of prednisone or equivalent at 6 months was 231 mg. No significant differences were observed in saliva cortisol measurements at 6 months between these 23 patients who started glucocorticoids and those who did not.

Sampling procedures and Assays

Saliva cortisol measurements

Saliva sampling was performed at home with +/-20 minutes variation of the prespecified time-point of the sampling, as instructed. The patients were provided with an illustrated step-by step guide of how to perform the saliva sampling procedure (available upon request).

Measurements were performed in several runs within 2 weeks after sampling due to restrictions in the long-term storage of saliva samples. Our laboratory has investigated the transferability of the reference range reported in the manufacturers' insert to our population by measuring saliva cortisol samples from 139 apparently healthy individuals aged 21 years and older at four time points during a day. Exclusion criteria were pregnancy, lactation, use of oral contraceptives and medication with cortisone/cortisol. The following reference values (95th percentile) were determined and no statistical difference was observed between males and

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females. Morning hours (6 am -12-noon.): <21.5 nmol/L; Afternoon hours (4-8 pm): <6.6 nmol/L; Late night hours (10-12 pm); 5.7 nmol/L.

Plasma ACTH measurements

Blood sampling for ACTH measurements was performed one day before the saliva sampling at the clinic with a maximal variation of 2 hours (between 7.30 and 9.30 am) on EDTA tubes. After venipuncture the tubes were immediately immersed in an ice bath, and the centrifugation to separate plasma from cells was strictly performed within one hour after venipuncture in a refrigerated centrifuge. Plasma samples were then stored at -80 ºC until further analysis. All plasma ACTH measurements were performed in one run.

The reference values of the assay, provided by the manufacturer were as follows: morning measurement: 1.9-11.4 pmol/L.

Results from sub-group analyses

Glucocorticoid-experienced patients displayed lower evening and night saliva cortisol measurements compared to glucocorticoid-naive patients **(Suppl. Table 1).** However, when accounting for differences in the disease duration the results no longer exhibited statistical significance (regression coefficient: 2.16, 95%CI: -0.76/5.1; and regression coefficient: 2.46, 95%CI: -0.52/5.4, respectively), probably highlighting an effect of the disease duration rather than a direct effect of the previous glucocorticoids treatment on the adrenal functional reserve.

Suppl. Table 1. Comparisons of plasma ACTH and diurnal saliva cortisol levels between RA patients and healthy controls after adjustments for differences in the sex ratio between the 2 groups

	Regression coefficient	95% CI	P-value
Plasma ACTH (pmol/L)	0.2	-1.4 to 1.8	0.794
Saliva Cortisol, 8am (nmol/L)	-0.1	-3.8 to 3.5	0.946
Saliva Cortisol, 12-noon (nmol/L)	-0.1	-1.6 to 1.5	0.918
Saliva Cortisol, 6pm (nmol/L)	1.3	-0.2 to 2.9	0.089
Saliva Cortisol, 10pm (nmol/L)	1.9	0.5 to 3.4	0.009
Time integrated daily saliva cortisol production-AUC	9.9	-8.2 to 28	0.278

The results presented are based on a linear regression model using sex as a confounding independent variable, in order to adjust for differences in the number of females between RA patients and healthy controls.

RA, Rheumatoid Arthritis; ACTH, adrenocorticotropic hormone; AUC, area under the curve; CI, confidence interval

Suppl. Table 2. Baseline saliva cortisol levels in seronegative versus seropositive RA patients

	Seronegative RA patients (n=13)	Seropositive RA patients (n=15)	P-value
Saliva Cortisol, 8am (nmol/L)	13.9 ± 7.8	16.3 ± 7.6	0.185
Saliva Cortisol, 12-noon (nmol/L)	5.3 ± 1.9	5.9 ± 3.2	0.856
Saliva Cortisol, 6pm (nmol/L)	4.3 ± 4.4	4.0 ± 2.8	1.000
Saliva Cortisol, 10pm (nmol/L)	4.0 ± 3.0	3.9 ± 4.0	0.821
Time integrated daily saliva cortisol production -AUC	83.8 ± 38.0	90.2 ± 38.3	0.717

Values are shown as mean ± SD. Mann–Whitney U test was performed for comparisons between groups, as applicable.

RA, Rheumatoid Arthritis; AUC, area under the curve

Suppl. Table 3. Baseline saliva cortisol levels in glucocorticoid-experienced versus glucocorticoid-naïve RA patients

	Glucocorticoid-experienced RA patients (n=15)	Glucocorticoid- naïve RA patients (n=13)	P-value
Saliva Cortisol, 8am (nmol/L)	13.4 ± 6.3	17.2 ± 8.7	0.294
Saliva Cortisol, 12-noon (nmol/L)	5.3 ± 2.2	6.0 ± 3.1	0.751
Saliva Cortisol, 6pm (nmol/L)	2.9 ± 2.3	5.6 ± 4.3	0.025
Saliva Cortisol, 10pm (nmol/L)	3.0 ± 3.2	5.1 ± 3.6	0.005
Time integrated daily saliva cortisol production -AUC	73.8 ± 24.3	102.7 ± 44.8	0.052
Disease duration, months	117 ± 117	28 ± 56	0.002

Values are shown as mean ± SD. Mann–Whitney U test was performed for comparisons between groups, as applicable. RA, Rheumatoid Arthritis; AUC, area under the curve

Suppl. Table 4. Circulating cortisol and ACTH levels at baseline in patients who did not achieve minimal disease activity at 6 months versus the rest of the RA patients

	Patients not achieving minimal disease activity (n=10)	Patients achieving minimal disease activity (n=10)	P-value
Serum Cortisol (nmol/L)	257 ± 88	300 ± 109	0.345
Plasma ACTH (pmol/L)	4.0 ± 1.8	2.4 ± 0.4	0.023
Cortisol/ACTH ratio	72 ± 26	128 ± 45	0.004
Time-integrated daily saliva cortisol production -AUC	64 ±17	102 ± 51	0.051

Values are shown as mean ± SD. Independent samples t-test (with or without Welch's correction) or Mann–Whitney U test was performed for comparisons between groups, as applicable.
RA, Rheumatoid Arthritis; ACTH, adrenocorticotropic hormone; AUC, area under the curve

Suppl. Table 5. Changes in diurnal saliva cortisol production, stress and depression rating scales and disease activity over time

	Baseline	3 months	6 months	P-value
Saliva cortisol, 8am (nmol/L)				
MDA at 6 months	18.4 ± 8.2	17.0 ± 8.3	14.7 ± 6.5	0.789
Non-MDA at 6 months	10.9 ± 4.0	13.9 ± 5.7	10.5 ± 4.5	0.368
Whole cohort	15.2 ± 7.6	15.7 ± 7.4	12.9 ± 6.0	0.420
Saliva cortisol, 12-noon (nmol/L)				
MDA at 6 months	5.4 ± 2.5	5.3 ± 2.2	6.8 ± 6.1	0.751
Non-MDA at 6 months	6.0 ± 2.9	5.5 ± 1.8	5.3 ± 2.7	0.490
Whole cohort	5.6 ± 2.6	5.4 ± 2.0	6.2 ± 4.9	0.512
Saliva cortisol, 6pm (nmol/L)				
MDA at 6 months	5.2 ± 4.2	4.0 ± 2.6	3.6 ± 1.8	0.355
Non-MDA at 6 months	2.7 ± 2.0	3.8 ± 2.2	2.4 ± 1.2	0.237
Whole cohort	4.2 ± 3.6	3.9 ± 2.4	3.1 ± 1.7	0.190
Saliva cortisol, 10pm (nmol/L)				
MDA at 6 months	3.7 ± 2.7	2.5 ± 1.1	3.0 ± 2.2	0.624
Non-MDA at 6 months	4.4 ± 4.5	2.1 ± 0.9	3.0 ± 3.8	0.228
Whole cohort	4.0 ± 3.5	2.4 ± 1.0	3.0 ± 2.9	0.223
Time integrated daily saliva cortisol, (AUC)				
MDA at 6 months	97 ± 41	86 ± 36	88 ± 45	0.424
Non-MDA at 6 months	74 ± 29	76 ± 18	65 ± 24	0.717
Whole cohort	87 ± 38	82 ± 30	78 ± 39	0.676
Perceived stress scale (Version PSS-14), severe, n				
MDA at 6 months	11/16	5/16	5/15	0.324
Non-MDA at 6 months	8/12	7/12	5/11	0.211
Whole cohort	19/28	12/28	10/26	0.208
Hamilton rating scale, moderate/severe, n				
MDA at 6 months	6/16	5/16	5/15	0.131
Non-MDA at 6 months	8/12	9/12	9/11	0.243
Whole cohort	14/28	14/28	14/26	0.650
Serum CRP levels (mg/L)				
MDA at 6 months	**23 ± 25**	**2.8 ± 3.3**	**2.0 ± 1.8**	**0.002**
Non-MDA at 6 months	30 ± 48	7.3 ± 8.7	3.9 ± 3.3	0.154
Whole cohort	**26 ± 36**	**4.5 ± 6.2**	**2.8 ± 2.7**	**<0.001**
DAS28-ESR				
MDA at 6 months	**5.3 ± 1.1**	**2.7 ± 1.0**	**2.1 ± 0.6**	**<0.001**
Non-MDA at 6 months	**5.5 ± 0.9**	**4.0 ± 1.1**	**3.6 ± 0.5**	**0.002**
Whole cohort	**5.4 ± 1.0**	**3.2 ± 1.2**	**2.7 ± 0.9**	**<0.001**

Values are shown as mean ± SD. Categorical data are shown as absolute number.
Repeated measurements ANOVA or Friedman test was performed for comparisons between related measurements, as applicable. The Cochran-Mantel-Haenszel (CMH) test was used for comparisons of multiple related measurements of categorical data.
MDA, minimal disease activity; AUC, area under the curve; PSS, perceived stress scale; CRP, C-reactive protein; ESR, Erythrocyte sedimentation rate