The Effect of Etanercept Therapy on Adrenal Steroid Metabolism in Juvenile Idiopathic Arthritis: A Steroid Metabolomics Approach

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Abstract

Objective

To evaluate the impact of anti-tumor necrosis factor-alpha (TNF α : etanercept [Enbrel®]) on adrenal activity in the acute phase of juvenile idiopathic arthritis (JIA) during which there is a decrease in adrenal androgen and cortisol levels.

Method

Eleven JIA patients aged 12 ± 6.2 years with a disease duration of 6.3 ± 5.2 years were enrolled. They were treated once weekly with etanercept (0.8 mg/kg) for 3 ± 2.8 years. Urine samples for gas chromatography-mass spectrometry steroid hormone analysis were collected before, and 1 and 3 days after etanercept injection and compared to age- and sex-matched healthy control.

Results

The levels of 23 of the 35 metabolites were low before etanercept treatment. Those 23 metabolites included 2 C₁₉ steroids (androgens), 3 C₂₁ steroid hormone intermediates, 14 cortisol metabolites, 2 corticosterone metabolites and 2 C₁₈ steroids (estrogens). One day post-treatment, only 5 of the 23 metabolite levels remained low. They included 2 C₁₉ metabolites, 2 C₂₁ steroid metabolites and 1 cortisol metabolite b-c. Three days post-treatment, the only metabolite levels that continued to be low were 1 C₁₉ metabolite, 2 C₂₁ steroid hormone intermediates and 1 cortisol metabolite (a-Cl), while the remaining 18 metabolites had already normalized after 1 day. DHEAS and 17 OH pregnenolone metabolite levels were the last ones to recover. Urinary metabolite ratios reflecting CYP21 and 11 β -HSD2 enzymatic activities were lower in JIA patients than in controls.

Conclusion

Almost all of the pre-etanercept treatment cortisol urinary metabolite levels were significantly lower than normal, and almost all rose to normal values by 1 day post-treatment. Blocking TNF α restores adrenal function in JIA.

Key Messages

What is already known about this subject?

- Serum cortisol concentrations are disproportionally low relative to the level of inflammation in rheumatoid arthritis (RA) and other inflammatory diseases.
- Tumor necrosis factor-alpha (TNF α) is elevated in the serum and the synovial fluid in children with juvenile idiopathic arthritis (JIA).
- Anti-TNF α treatment has an effect on the hypothalamic–pituitary–adrenal axis and improves adrenal hormone secretion in adults with RA. Comparable findings were never confirmed in pediatric JIA patients.

What does this study add?

- Anti-TNF α treatment has a rapid effect on urine adrenal metabolites in children with JIA.

- The therapeutic effect of anti-TNF treatment in JIA may be related to its effect on the restoration of adrenal function and cortisol levels.

How might this impact on clinical practice or future developments?

- The current study introduces the concept that the urinary steroid metabolome is suitable for assessing and monitoring the disease activity in JIA as well as for predicting disease flare.

Introduction

Juvenile idiopathic arthritis (JIA) is the most common rheumatic disease in children and a major cause of functional disability. The chronic inflammatory synovitis and systemic features of JIA are mediated by the cytokine products of an activated immune system. Among the many cytokines that are involved in the acute phase, the levels of interleukin-6 (IL-6), IL-8 and tumor necrosis factor alpha (TNF α) are the most predominantly elevated. TNF α is one of the proinflammatory cytokines that has a complex role in the pathogenesis of rheumatoid arthritis (RA). [1][2]. It is also elevated in the serum and in the synovial fluid in children with juvenile rheumatoid arthritis (JRA) [3].

Cortisol is a major anti-inflammatory substance whose low levels during the evening and night are linked to an increase in proinflammatory cytokines, such as TNF α and IL-6, during the early morning. That rise in cortisol levels in the early morning is related to the inhibition of inflammation by cytokines during the day [4] This cortisol circadian rhythm is clinically linked to pain and stiffness, which are prominent during the early morning in RA. Several studies have observed that serum cortisol concentrations in RA and other inflammatory diseases are disproportionally low relative to the level of inflammation [5]. The hypocortisolemia in JIA may result either from primary or secondary hypoadrenalism. The endocrine profile in some untreated RA patients revealed elevated adrenocorticotrophic hormone (ACTH) levels without hypercortisolemia, a combination that reflects resistance to ACTH or the presence of primary hypoadrenalism [6][7]. These observations correspond with a seminal study which showed that TNF α is a potent inhibitor of adrenocorticotropin-induced cortisol production and steroidogenic P450 enzyme gene expression in cultured human fetal adrenal cells.[8] Other studies in patients with active JIA showed low levels of ACTH, serum cortisol and urinary free cortisol, a combination that reflects a disturbance in the hypothalamic pituitary adrenocortical axis (HPA).

Etanercept (Enbrel®, Immunex, Seattle), a genetically engineered fusion protein consisting of 2 identical chains of the recombinant extracellular human TNF-receptor p75 monomer fused with the Fc domain of human immunoglobulin1, effectively binds TNF α and lymphotoxin- α and inhibits their activity. Treatment with etanercept reportedly leads to significant improvement in patients with active polyarticular JRA [8].

The aim of this study was to evaluate the role of etanercept on adrenal steroid hormone metabolism by assessing the urinary steroid metabolome in JIA.

Methods

Patients

The study group was composed of all consecutive patients who were treated with once weekly etanercept injections and followed in the Pediatric Rheumatology Service in Rambam Hospital (Haifa, Israel), all methods were performed in accordance with the relevant guidelines and regulations. An age- and sex-matched healthy control was matched to each patient. The diagnosis of JIA was made according to the JIA criteria of the International League of Associations for Rheumatology classification [9]. Disease severity was evaluated by measuring the number of active joints and C-

reactive protein (CRP) levels. Patients who were treated with corticosteroids by oral or intra-articular injection during the preceding 3 months were excluded.

Methods

Urinary samples were obtained from all patients. The first sample was collected on the day before Enbrel injection. The second and the third samples were collected one day and three days after the injection. One urine sample was obtained from healthy controls. All urine samples were spot urines from first morning voiding.

Urinary steroid metabolites were analyzed by using quantitative data that were generated by gas chromatography-mass spectrometry (GC-MS) analysis as described previously[10]. In brief, free and conjugated urinary steroids were extracted by solid phase extraction, and conjugates were enzymatically hydrolyzed. After recovery of hydrolyzed steroids by solid phase extraction, known amounts of internal standards (5 α -androstane-3 α , 17 α -diol, stigmasterol) were added to each extract before formation of methyloxime-trimethylsilyl ethers. GC was performed using an Optima-1 fused silica column housed in an Agilent Technologies 6890 series GC that was directly interfaced to an Agilent Technologies 5975 mass selective detector. Injections were made into an 80°C (2 min) oven, in which the temperature was increased by 20°C/min to 190°C (1 min). To separate steroids the temperature was subsequently increased by 2.5°C/min to 272°C. For each analyte the monitoring of two typical fragment ions (target ion and qualifier ion) ensured specificity. Quantitation took place in the linear range of the calibration plots of our analytes. Values of 35 metabolites identified by GC-MS were compared between JIA patients and healthy controls. This generated a metabolic profile of JIA patients before and after Enbrel treatment compared to healthy controls.

Statistical analysis

The results are expressed as mean $\pm$ standard deviation for data showing normal distribution, and as median for data showing abnormal distribution. The paired T-test and the paired Wilcoxon test were applied to compare the data showing and not showing normal distribution of the repeated specimens, respectively, with that of the controls. Fisher's exact test was used to compare the number of metabolites whose values were significantly different from the control values, after which Bonferroni correction for multiple comparisons was applied. Significance was determined at p values <0.05.

Results

Eleven JIA patients were enrolled into the study. There were 8 females and 3 males with an age range from 3 to 21 years. Five patients had polyarthritis, 3 had oligoarthritis, 2 had psoriatic arthritis, and 1 had systemic arthritis. The disease duration ranged from 2 to 18 years. The duration of etanercept treatment ranged from 6 months to 10 years. Two patients were also treated with methotrexate during the study (patients 1 and 2, **table 1**).

Thirty-five urinary metabolites that provide an integrated picture of human steroid hormone metabolism were analyzed before and after etanercept treatment (Fig -1). The levels of 23 of those 35 metabolites were significantly lower than normal before treatment. The 23 metabolites included 4 of the 11 C₁₉ steroids (androgens), 5/8 C₂₁ steroid hormone intermediates, 10/10 cortisol metabolites, 2/3 corticosterone metabolites and 2/3 C₁₈ steroids (Table 2). One day after etanercept treatment, only five out of the 23 metabolites remained significantly lower than normal in the JIA patients. Those five metabolites were 2 C₁₉ metabolites, androsterone (An) and 5 α -androstane-3 α , 11 β -diol-17-one (11OH-An) and 2 C₂₁ steroids [pregnanediol (P5D preg) and 11-O-Pt (5 β -Pregnane-3 α , 17 α , 20 α -triol-11-on)] (Table 2). Three days after etanercept treatment, only five metabolites were significantly low: 2 C₁₉ steroid metabolites An and 11OH-An, 2 C₂₁ intermediates Pt and P5D and 1 cortisol metabolite (a-Cl).

None of the metabolites at any time during the collection was significantly higher in the JIA patients than in the control group. The metabolite ratios that reflect enzyme activities before etanercept treatment and 3 days following it are presented in Table 3. The ratio $(\text{THE}+\text{THF}+\text{aTHF})/\text{PT}$ that reflects the activity of CYP21 reached a p value of 0.06, and the ratio of $((\text{a-C})+(\text{b-C}))/((\text{a-Cl})+(\text{b-Cl}))$ that reflects the activity of the renal $11\beta\text{-HSD2}$ reached a p value of 0.07.

Discussion

JIA is the most common rheumatic disease in children, with an incidence of 1.6-23 cases/100,000 [11]. The chronic inflammatory synovitis and systemic features of JIA are mediated by cytokine products of an activated immune system, including the TNF α , IL-1 and IL-6 axis. Anti-TNF is an effective therapy for modifying the course of JIA, and one that is usually administered as a second-line agent in treating patients with JIA [8].

Bilginer et al showed that JIA patients with active disease have low early morning serum cortisol levels as well as low ACTH levels that correlate with high IL-6 levels as seen in active disease [12], representing a combination that can reflect hypophysitis or possible hypothalamic involvement. In line with that observation, the findings that urinary-free cortisol levels in active JIA patients are lower than those in healthy controls and that they rise during periods of remission in parallel to the serum cortisol levels suggest that the activity of the HPA axis is affected during the active phase of JIA [12][13]. During remission, JIA patients reportedly have normal cortisol levels in the presence of high ACTH levels, a finding that may suggest partial resistance to ACTH [14].

TNF has an important role in the pathogenesis of JIA. Patients with JIA have high levels of TNF α in inflamed joints, and anti-TNF is a very potent therapy in JIA as it is in RA. Atzeni et al showed that anti-TNF therapy in adult RA patients normalized the HPA axis by increasing the ACTH and cortisol levels and by decreasing the ratio of ACTH to cortisol [15]. Those findings suggest that anti-TNF treatment has an effect on the HPA axis and that it improves adrenal hormone secretion, but comparable findings have never been reported in pediatric JIA patients [15][15][14][13][13][15].

The aim of the current study was to evaluate the role of anti-TNF therapy on the pediatric adrenal steroid hormone metabolism as reflected by urinary steroid metabolites determined by GC-MS. We found that 23 urine metabolite levels were significantly lower in JIA patients before they underwent etanercept treatment: 18 of them normalized 1 day post-treatment and only 5 remained lower than normal after 3 days. Interestingly, the levels of the 5 An and 11OH An C₁₉ metabolites, Pt and P5D (that are C₂₁ metabolites) and a-Cl (that is a cortisol metabolite) remained lower than normal 3 days after the treatment.

Jäättelä et al seminal study (8) showed that TNF α inhibits the expression of mRNAs of adrenal cytochrome P450 oxidases, P450scc (cholesterol side-chain cleavage enzyme/20.22-desmolase), P450c11 (11 beta-hydroxylase/18-hydroxylase/18-methyl oxidase), P450c17 (17 alpha-hydroxylase/17, 20-lyase) and P450c21 (21-hydroxylase) in human fetal adrenals. The decrease in gene expression was accompanied by a decrease in cortisol but not in either dehydroepiandrosterone sulphate (DHEAS) or in androstenedione. All of the effects of TNF α were neutralized by the addition of monoclonal anti-TNF α antibody. These results demonstrated that TNF α suppresses the synthesis of cortisol and shifts the steroid secretory pattern towards androgen production, at least partially, by suppressing the accumulation of mRNAs for adrenal cytochrome P450 oxidases.

The estradiol metabolite levels were low in the 8 females before the administration of etanercept treatment. Given that testosterone is aromatized to estrogen by Cyp19, those low levels can be related to low testosterone levels produced by the effect of TNF α on P450c17 (17 alpha-hydroxylase/17, 20-lyase), and to the fact that TNF inhibits the conversion of DHEAS to DHEA in RA synovial cells [16].

The finding that four urine metabolites (An, Pt, P5D and a-Cl) remained significantly low 3 days after etanercept treatment may indicate that the enzymes that are involved in their metabolism are more affected by TNF α than the other enzymes. Urine metabolite ratios reflect enzymatic activities. There was no significant difference in most of those ratios between the control group and the patient group before and after etanercept treatment, whereas the ratios that reflect 21 hydroxylase and 11 β -hydroxysteroid dehydrogenase (11 β -HSD) type 2 activity were very close to being significantly different between the groups. That finding indicates that these 2 enzymes were more affected by Etanercept in the patient group. Notably, these 2 enzymes are involved in cortisol production and cortisol conversion to cortisone. Active cortisol is converted to inactive cortisone mainly by the kidney via 11 β -hydroxysteroid dehydrogenase (11 β -HSD) type 2 in order to protect the nonspecific mineralocorticoid receptor from activation by cortisol. The liver is the major organ for converting inactive cortisone to active cortisol, and it does so via 11 β -HSD1. The expression of 11 β -HSD1 is enhanced by TNF and proinflammatory cytokines. The cytokine-induced increased expression of 11 β -HSD1 is mainly involved at the intracellular compartment, however, it can induce a change in the HPA axis [17].

Although most of the patients in the current study were either free of active disease or had mild disease activity, their urine cortisol metabolites were nevertheless low compared to the normal controls. One of the challenges in treating JIA patients is to decide when to stop treatment, such as anti-TNF, and to try to predict who will need to resume therapy. Toward this end, the interrupted HPA axis in JIA patients with active as well as subclinical disease may be able to identify the patients for whom it is safe to stop therapy, given that the recovery of the HPA axis improves not only the inflamed joints but also other important health aspects, such as growth, bone health and general wellbeing.

Our study has a few limitations that bear mention. The main limitation is our small number of patients, and another is their range of ages and different stages of sexual development that may influence the HPA axis. We tried to overcome this latter limitation by matching them with a control group by age and Tanner stage development. Another drawback is that we do not have 24-hour urinary samples to precisely calculate the excretion rates of urinary steroid hormone metabolites and thereby assess hormonal production rates. Although our study population is limited, we were able to show that most of patients with JIA have an interrupted HPA axis and that anti-TNF therapy was able to correct it.

Conclusions

The findings of the current study demonstrated that anti-TNF α treatment has a rapid effect on urine adrenal metabolites in children with JIA. The therapeutic effect of anti-TNF treatment in JIA may be related to its effect on the restoration of adrenal function and cortisol levels. We propose that the urinary steroid metabolome is suitable for assessing and monitoring the disease activity in JIA as well as for predicting disease flare. A larger study is warranted to confirm our findings and to evaluate whether the urinary steroid metabolome can be used as a predictor to assess and monitor disease activity and serve as a predictor for disease flare.

- Subheading- Anti-TNF α treatment has a rapid effect on urine adrenal metabolites in children with JIA. The therapeutic effect of anti-TNF treatment in JIA may be related to its effect on the restoration of adrenal function and cortisol levels. steroid metabolome is suitable for assessing and monitoring the disease activity in JIA as well as for predicting disease flare.

Abbreviations Of Steroids

An	5 α -Androstane-3 α -ol-17-one (Androsterone)
Et	5 β -Androstane-3 α -ol-17-one (Etiocolanolone)
DHEA	5-Androstene-3 β -ol-17-one (Dehydroepiandrosterone)
16 α -OH-DHEA	5-Androstene-3 β ,16 α -diol-17-one
A5-3 β ,17 α a	5-Androstene-3 β ,17 α -diol diol
A5-3 β ,17 β	5-Androstene-3 β ,17 β -diol (Androstenediol-17 β)
A5T- 16 α	5-Androstene-3 β ,16 β ,17 β b-triol (Androstenetriol-16 α)
PD	5 β -Pregnane-3 α ,20 α -diol (Pregnanediol)
PT	5 β -Pregnane-3 α ,17 α ,20 α -triol (Pregnanetriol)
P5D	5-Pregnene-3 β ,20 α -diol (Pregnanediol)
P5T-17 α	5-Pregnene-3 β ,17 α ,20 α -triol (Pregnenetriol-17 α)
Po-5 β ,3 α	5 β -Pregnane-3 α ,17 α diol-20-one (17 α -OH-Pregnanolon)
Po-5 α ,3 α	5 α -Pregnane-3 α ,17 α -diol-20-one
F	4-Pregnene-11 β ,17 α ,21-triol-3,20-dion (Cortisol)
THE	5 β -Pregnane-3 α ,17 α ,21-triol-11,20-dion (TH-Cortisone)
THF	5 β -Pregnane-3 α ,11 β ,17 α ,21-tetrol-20-one (TH-Cortisol)
α -THF	5 α -Pregnane-3 α ,11 β ,17 α ,21-tetrol-20-one (Allo-TH-Cortisol)
α -Cl	5 β -Pregnane-3 α ,17 α ,20 α ,21-tetrol-11-one (α -Cortolone)
β -Cl	5 β -Pregnane-3 α ,17 α ,20 β ,21-tetrol-11-one (β -Cortolone)
α -C	5 β -Pregnane-3 α ,11 β ,17 α ,20 α ,21-pentol (α -Cortol)
β -C	5 β -Pregnane-3 α 11 β ,17 α ,20 β ,21-pentol (β -Cortol)
6 β -OH-F	4-Pregnene-6 β ,11 β ,17 α ,21-tetrol-3,20-dion
20 α -DHF	4-Pregnene-11 β ,17 α ,20 α ,21-tetrol-3-one
11-OH-An	5 α -Androstane-3 α ,11 β -diol-17-one
11-O-An	5 α -androstane-3 α -ol-11,17-dion
11-OH-Et	5 β b-androstane-3 α ,11 β -diol-17-one
11-O-PT	5 β -Pregnane-3 α ,17 α ,20 α -triol-11-one
THA	5 β -Pregnane-3 α ,21-diols-11,20-dion
THB	5 β -Pregnane-3 α ,11 β ,21-triol-20-one (TH-Corticosterone)
α -THB	5 α -Pregnane-3 α ,11 β ,21-triol-20-one (Allo-TH-Corticosteron)
THS	5 β -Pregnane-3 α ,17 α ,21-triol-20-one
E1	Estrone
E2	Estradiol

E3

Estriol

T

Testosterone

Declarations

Ethics approval and consent to participate :REB approval for the study was received from Rambam Helsinki Committee (RMB) and all patients signed the informed consent for.

Consent for publication-Not applicable

Availability of data and materials-The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests-The authors declare that they have no competing interests

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Authors' contributions: YBA wrote the first draft of the manuscript and design the study, AK conduct the study, help to design the study and analyze the data, MFH- analyzed and interpreted the patient data conduct the laboratory study and was a major contributor in writing the manuscript SAW- analyzed and interpreted the patient data and was a major contributor in writing the manuscript DT- design the study and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1 Clinical characteristic of the patients with juvenile idiopathic arthritis

	Sex	Age	JIA subtype	Disease duration (years)	No. of active joints	CRP levels (mg/dl)*	Treatment duration with etanercept (years)	Etanercept dosage (mg*1/w)
1	F	16	Psoriatic arthritis	3	0	3.6	0.7	50
2	F	16	Oligoarthritis	4	0	0.5	3	50
3	M	10	Polyarthritis	9	0	4.5	0.8	25
4	F	21	Oligoarthritis	18	3	n/a	10	50
5	M	8	Polyarthritis	3	1	5.5	3	25
6	F	15	Polyarthritis	7	0	8.6	6	50
7	F	18	Oligoarthritis	15	0	n/a	5	50
8	M	5	Systemic	4	0	0	3	25
9	F	15	Polyarthritis	4	0	10.1	3	50
10	F	15	Psoriatic arthritis	6	1	0	1	50
11	F	3	Polyarthritis	2	0	0	0.6	7.5

n/a, not available

Table 2 Urine metabolite values in the juvenile idiopathic arthritis patients and controls

Metabolite	Before etanercept		One day after etanercept		Three days after etanercept		Control group
	Median, (Min-Max) µg/L	p values before vs controls	Median, (Min-Max) µg/L	p values 1 day after vs controls	Median, (Min-Max) µg/L	p values 3 days after vs controls	Median, (Min-Max) µg/L
C19-steroids (androgens)							
An	427.81 (17.97-2054.22)	0.003	575.66 (13.45 - 1644)	0.019	412.92 (16.73- 4449.08)	0.008	1192.54 (81.57- 4116.32)
Et	392.54 (24.68- 3063.66)	0.09	513.98 (11.30 4616.94)	0.4	489.91 (14.445773.05)	0.4	1145.88 (87.36- 3525.82)
DHEA	392.54 (24.68-3063.66)	0.8	41.73 (0- 2741.18)	0.8	23.24 (0-926.18)	0.4	94.03 (10.65- 2582.57)
16a-OH- DHEA	25.79. (0-1536.30)	07	92.51 (0- 1621.95)	0.9	125.21 (0- 1634.31)	0.8	272.12 (.00- 872.27)
A5-3b,17a	14.12 (0-85.72)	0.0049	14.94 (0-159.06)	0.07	19.03 (.00-175.92)	0.2	45.22 (11.03- 112.41)
A5-3b,17b	19.52 (0-77.32)	0.3	19.34 (0-85.83)	0.31	27.58 (.00-106.72)	0.3	41.18 (.00- 358.93)
A5T-16a	87.25 (0-1200.50)	0.9	81.11 (0- 1548.20)	0.9	113.05 (.00-2719.29)	0.6	147.46 (39.50- 566.38)
11-OH-An	276.47 (34.45-637.49)	0.0053	193.16	0.039	241.12	0.047	579.13

					(39.88- -781.14)		(44.47- 1281.82)		(169.17- 1059.13)
11-O-An	24.75 (10.70-89)	34.45	637.49	0.028	21.35 (8.53- -70.90)	0.1	26.16 (9.02-134.42)	0.09	40.98 (22.13- 150.96)
11-OH-Et	57.53 (9.48-988.60)			0.2	134.69 (7.29- 1608.09)	0.5	64.33 (7.81-2263.65)	0.68	354.46 (58.94- 872.17)
T				0.89		0.34		0.3	
C21-steroids Pregnenolone and progesterone metabolites									
PD	86.29 (0-827.49)			0.4	53.86 (14.87- 995.09)	0.5	71.37 (.00-839.30)	0.3	207.19 (49.24- 788.44)
PT	306.98 (34.91-849.41)			0.007	221.14 (27.86- 2436.86)	0.2	322.28 (32.96-1692.6)	0.001	565.08 (144.02- 1625.78)
P5D	72.93 (0-226.63)			0.015	38.94 (0- -291.06)	0.013	44.69 (00-156.34)	0.009	143.69 (65.56- 484.74)
P5T-17a	105.06 (22.56-529.14)			0.11	91.35 (14.28- 797.18)	0.3	123.81 (17.59-793.84)	0.2	292.11 (65.21- 865.44)
Po-5b,3a	41.27 (12.02-531.93)			0.0001	28.40 (11.67- 672.43)	0.08	53.95 (18.14-218.24)	0.1	86.05 (42.16- 600.95)
Po-5a,3a	9.17 0-17.69)			0.064	7.08 (0-21.84)	0.1	9.70 (.00-29.03)	0.1	12.19 (7.73- 83.24)
11-O-Pt ...	5.14 (2.39-41.76)			0.0018	3.98 (2.21- 15.49)	0.03	4.16 (2.21-83.40)	0.8	9.22 (4.42- 49.41)

THS	55.47 (24.89-211.36)	0.0065	57.22 (24.71-336.07)	0.1	70.18 (27.00-266.76)	0.16	104.33 (37.82-295.79)
Cortisol metabolites							
F	40.21(20.62-134.45)	0.025	35.64 (19.75-266.41)	0.7	44.20 (24.59-219.24)	0.5	74.27 (28.16-143.60)
THE	2467.00 (534.81-5709.12)	0.022	1881.51 (541.44-11880.03)	0.6	1960.83 (668.60-11234.45)	0.2	3396.78 (831.73-7896.67)
THF	871.67 (205.93-1959.04)	0.028	620.38 (305-3295.74)	0.4	838.12 (366-667.05)	0.3	1097.69 (420.88-2403.82)
a-THF	434.13 (47.59-1194.49)	0.016	552.75 (61.32-1942.16)	0.3	493.77 (46.30-2137.91)	0.1	902.28 (93.23-1488.69)
a-Cl	715.04 (254.56-1823.31)	0.012	643.37 (274.96-4126.42)	0.6	666.16 (351.31-2702.04)	0.03	1246.88 (598-2701)
b-Cl	388.41 (68.26-1128.53)	0.001	447.54 (190-1435)	0.2	461.83 (58.38-1641.23)	0.08	705.23 (126.71-1402.16)
a-C	86.79 (38.58-241.82)	0.002	85.60 (37.80-458.59)	0.07	99.09 (46.28-489.07)	0.08	182.45 (52.43-390.19)
b-C	252.81 (55.43-648.28)	0.014	298.91 (70.29-801.36)	0.045	248.15 (45.41-894.96)	0.07	513.62 (105.74-1615.79)
6b-OH-F	87.86 (49.05-312.35)	0.02	94.19 (00-64.50)	0.1	115.32 (51.3-82)	0.5	277.13 (69.52-503.68)
20a-DHF	25.93 (0-55.62)	0.03	23.54	0.1	22.79 (.00-103.23)	0.3	40.27

			(.00- 113.42)				(.00- 93.06)
Corticosterone metabolites							
THA	101.69 (31.18-273.00)	0.034	83.84 (25.20- 810.)	0.49	117.02 (45.77-396.07)	0.07	274.83 (46.04- 758.96)
THB	111.86 (32.22-445.00)	0.025	65.14 (.00- 631.76)	0.3	90.52 (52.72-393.93)	0.052	251.85 (81.56- 555.59)
a-THB	170.58 (26.20-360.73)	0.053	149.01 (23.98- -1426.28)	0.3	151.04(41.97- 749.04)	0.09	301.34 (39.35- 1411.08)
C18-steroids (estrogens)							
E1	0(0-28.04)	0.040	0 (0- 38.73)	0.3	0.00(0.00- 24.52)	0.19	18.33 (0- 33.65)
E2	0 (0-7.87)	0.03	0 (0-8.97)	0.1	6.00 (0-7.24)	0.3	5.77 (0- 13.41)
E 3	0(0-30.13)	0.29	0 (0- 42.68)	0.2	5.77 (0-13.41)	0.4	0.00 (0.00- 95.86)

Significant values (p<0.05) appear in **bold**

Min, minimum; Max, maximum

Table 3 Urine metabolites ratios that reflect enzyme activities

Enzyme	Ratio	p value
21-hydroxylase	THE+THF+aTHF)/PT	0.06
17-hydroxylase	(THE+THF+aTHF)/(THA+TH B+aTHB)	0.39
	(An+Et)/(THA+THB+aTHB)	0.57
11b-hydroxylase	(THE+THF+aTHF)/100*THS:	0.32
11b-hSD2	THE/ (THE +a THF)	0.53
	(a-Cl)+(b-Cl))/(a-C+ b-C)	0.38
11b-HSD1	(THF+aTHF)/THE:	0.95
(liver, adip)		
	(a-Cl+b-Cl) /(a-Cl+ b-Cl):	0.07
5 α-reductase	An/Et	0.28
	11-OH-An/11-OH-Et	0.65
	aTHB/THB	0.78
	aTHF/THF	0.89

Significant values (p<0.05) appear in **bold**

Figures

Figure 1

The black columns represent the percentage of significantly lower levels of adrenal metabolites in urine and the white columns represent the percentage of normal metabolites before and after etanercept treatment.