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Evaluation of tumor necrosis factor alpha production in ex vivo short term culture whole blood from women with polycystic ovary syndrome



Abstract

In mammals, the pleiotropic biological functions of tumor necrosis factor alpha (TNF- α) may include important effects on human reproductive physiology. Thus, chronic anovulation, oligo or amenorrhea, infertility, hyperandrogenism, obesity, insulin resistance and increased TNF α serum levels have been observed in women affected by polycystic ovary syndrome (PCOS). Whole blood short - term

cell cultures (WBSC) are simple systems where the capacity to produce TNF- α by circulating leukocytes, mainly of the macrophage/monocyte lineage, can be accurately quantified. Given the relevance of monocytes/macrophages in the production of TNF- α , in this study, in a control-case approach, WBSC from women with PCOS were analyzed in their basal and lipopolysaccharide (LPS)-stimulated capacity to produce the cytokine. These measurements did not correlate with the increased serum levels of the cytokine and the normal levels of cortisol, found in PCOS women. Increased serum TNF- α levels in PCOS women correlated positively with body mass index and negatively with insulin sensitivity. In spite of the increased serum TNF- α levels in PCOS women, basal and LPS stimulated production of the cytokine, by the ex vivo WBSC from these patients, were within normal values.

Full text

Introduction

Tumor necrosis factor alpha (TNF α) is one of the most pleiotropic cytokines in mammals and, among its wide array of biological functions, effects on regulation of ovarian function have been described [1, 2]. Thus, chronic anovulation, oligo or amenorrhea, infertility, hyperandrogenism, obesity, insulin resistance and higher TNF- α serum levels have been detected in women affected by polycystic ovary syndrome (PCOS), one of the most frequent endocrine disorders during the female reproductive age [3, 4].

Similar to other cytokines, TNF- α production can be swiftly activated by a variety of stimuli, even before an increase in glucocorticoid synthesis [5]. On the other hand, the interactions between the immune system and glucocorticoids are complex, resulting in activation or suppression of the immune response [6, 7].

Basal or lipopolysaccharide (LPS) stimulated TNF- α production by blood leukocytes, mostly of the macrophage / monocyte lineage, can be accurately measured in whole blood (*ex vivo*) short-term cultures (WBSC) [8, 9]. In normal humans, large inter-individual variations in TNF- α production in the WBSC system, following LPS stimulation, have been described [10-13]. This variability may be related with polymorphisms affecting either the TNF- α gene or its promoter [14-18]. Glucocorticoid levels and magnitude or opportunity of stimuli are also important factors affecting cytokine levels [19]. Thus, interactions

among the hypothalamus-pituitary-adrenal axis (HPA), the gonadal axis and stress, with consequent interruption of ovulatory cycles, have been reported [20-24]. Moreover, an association between hyperactivity of the HPA axis with chronic hypersecretion of corticotropin releasing hormone and insulin resistance has also been observed [25, 26].

Given the increased serum levels of TNF- α in women with PCOS and the possible involvement of the cytokine in this disease [2], in this study we asked whether this syndrome includes an altered capacity of monocytes / macrophages, basal or LPS stimulated, to produce the cytokine, using the WBSC *ex vivo* system.

Methods

Patients

Sixteen women, mainly of hispanic origin, aged 25.6 ± 4.5 yr (range 17 to 33), with a body mass index (BMI) 32.7 ± 5.7 kg/m², and waist/hip ratio (W/H) 0.89 ± 0.09 , were recruited among those consulting the Endocrinology, Gynecology and Infertility Units of our University Hospital. PCOS diagnosis was based in the history of oligo or amenorrhea, infertility, and clinical or biochemical hyperandrogenism. Hirsutism was clinically evaluated by the Ferriman-Gallwey score. A value over 6 was considered positive. The average of PCOS women was 14.3 ± 8.7 . Laboratory criteria of hyperandrogenism was established when the free androgen index (FAI) was greater than 4.5 and androstenedione levels were over 2.8 ng/ml. Three patients showed glucose intolerance in the 75 g oral glucose tolerance test. No women had received hormonal contraceptive therapy, metformine or troglitazone, during the last three months before the study.

Relevant biochemical parameters measured in PCOS women included: FAI (calculated from sex hormone binding globuline-SHBG/Total testosterone $\times 100$), androstenedione, basal 17-OH progesterone, dehydroepiandrosterone sulfate (DHEASO₄), total testosterone and prolactin (see Results Section).

Eleven healthy women, 26.7 ± 4 yr, BMI 23 ± 2.2 kg/m², W/H 0.65 ± 0.059 , Ferriman-Gallwey score 6 ± 2 , without hormonal contraceptive therapy or family

history of type 2 or gestational diabetes, with normal fasting glycemia and regular menstrual cycles, were selected as controls.

For at least three weeks, prior or during this study, all participating women did not receive therapies with antibiotics, non-steroidal antiinflammatory drugs, glucocorticoids or other relevant products. Absence of concomitant infectious conditions was estimated by standard clinical criteria.

Study protocol

To evaluate insulin resistance in PCOS women, an i.v. insulin tolerance test was performed, following standard procedures [27, 28]. After a 12 h overnight fast, an indwelling catheter was placed in a forearm vein between 8.00 and 9.00 am. A basal blood sample was obtained for glucose and insulin determinations. Then, 0.1 U/kg body weight of rapid action insulin was injected and blood samples were obtained at 0, 3, 6, 9, 12 and 15 minutes, for glucose measurement. The rate constant for plasma glucose disappearance (K_{ITT}) was $0.693/t_{1/2}$. Plasma glucose half-life ($t_{1/2}$) was calculated from the linear slope of the blood glucose concentration, from 3 to 15 minutes after insulin injection.

On a different day, a 10 ml blood sample was obtained from patients and controls, from a forearm vein, at 3.00 pm, in the absence of anticoagulant substances. In those women presenting menstrual cyclic bleeding, the samples were obtained between days 3 to 5 of the cycle. All sera were frozen at -20°C .

Serum levels of TNF- α and cortisol were measured in commercial chemoluminescent enzyme immunometric assays (Immulite, Diagnostic Products Corporation, Los Angeles, CA), with sensitivities close to 4 pg/ml and 1 mg/ml, respectively.

Glucose was determined by a glucose oxidase method, and insulin by the microparticles enzyme immunometric technique (AxSYM, Abbot).

Ex vivo short term culture of whole blood (WBSC)

As originally described [9], serum samples were obtained for basal TNF- α determinations under sterile conditions. One ml of blood was diluted with 4 ml of

RPML-1640 medium. The mixture, supplemented with L-glutamine (200 mM), penicillin (200 IU/ml), streptomycin (200 mg/ml) and heparin (2 IU/ml), was distributed in 360 ml aliquots in 24 well flat bottom plates (Nunc^R, U.S.A.). After 4 h incubation at 37 °C in 5% CO₂ atmosphere, sonicated LPS from *E. coli*, 026:B6 serotype (Sigma, St. Louis, Mo. U.S.A.) was added to a final concentration of 10 mg/ml. Controls included cultures in the absence of endotoxin and in the absence of blood. All samples were then incubated for 12 h and each well content was transferred to Eppendorf^R tubes and centrifuged at 1,000 rpm for 5 min. Approximately 4 ml of supernatant were collected from each sample and stored at - 70 °C.

Immunoradiometric assay (IRMA) for assessing TNF levels in WBSC

Performed as previously described in our laboratory [13]. briefly, polyvinylchloride (PVC) microtitration plates were sensitized with 3 mg/ml of a monoclonal IgM antibody (E8) anti human recombinant TNF (anti-hrTNF). The plates were incubated for 12 hrs at 4 °C and the remaining active sites were blocked with 1% w/v bovine serum albumin (BSA) in phosphate buffer (PBS-BSA 1%). A standard curve was generated with human recombinant TNF- α (hrTNF) (8 to 8,000 pg). All samples were incubated for 2 h at 4 °C. A polyclonal rabbit serum anti-hrTNF diluted 1/1,000 was added, followed by incubation for 2 h at 4 °C. After washing, approximately 1 ng (100,000 cpm) of a ¹²⁵I-labelled, affinity purified, goat IgG anti-rabbit IgG (g-chain specific) was added, and incubated for 2 hr at room temperature. All incubations were followed by four washes with PBS/NP40 0.05% v/v and the radioactivity bound to the solid phase was measured. The sensitivity of this assay was around 30 pg/ml.

Statistics

Results are expressed as mean $\pm$ SD. The Mann-Whitney statistic test was used to assess the significance of mean differences among the groups of subjects, with 95% confidence intervals. Correlations were determined by using the Spearman test and $p < 0.05$ was considered statistically significant.

Ethical Considerations

In agreement with the Helsinki declaration, regulations established by the University of Chile Clinical Hospital Ethics Committee were followed strictly in all procedures carried out with participating individuals, including an informed written consent form.

Results

Table 1 summarizes the biochemical characteristics of women with PCOS participating in this study. With the exception of slightly increased FAI and androstenedione levels, all other values were within normal ranges, ruling out other causes of hyperandrogenism and amenorrhea. All PCOS patients were overweight or android obese, and a waist/hip ratio (W/H) over 0.8 was found in 15 of 16 individuals. Laboratory findings of insulin resistance included hormone increases, both basal and 120 minutes after 75 g of oral glucose administration (21 ± 12 and 154 ± 125 mIU/ml, respectively). A decrease in K_{ITT} value ($4.35 \pm 1.3\%$) was also observed in most of the cases.

Table 1 Biochemical parameters in 16 PCOS patients

	$\bar{x} \pm DS$	Normal range
Glycemia _(basal) (mg/dl)	91 ± 8	60-110
Glycemia _(120 min)	113 ± 31	< 140
Insulin _(basal) (mIU/ml)	21 ± 12	≤ 20
Insulin _(120 min)	154 ± 125	≤ 60
K_{ITT} (%)	4.3 ± 1.3	≤ 5
Prolactin (ng/ml)	14.5 ± 5.8	3-30
LH/FSH	1.8 ± 1	≤ 2

17 OH Progesterone (ng/ml)	1.9 ± 1.1	0.3-4.0
DHEASO ₄ (mg/ml)	2.21 ± 0.92	0.35-4.3
Total Testosterone (nmol/L)	3.1 ± 0.7	0.7-3.1
SHBG (nmol/L)	33.4 ± 15.9	18-114
FAI	11 ± 5.4	< 4.5
Androstenedione (ng/ml)	4 ± 1	0.8-2.8

As summarized in *Table 2*, WBCS from PCOS women behaved normally, in their capacity to respond to LPS treatment. In other words, both WBSC from PCOS and normal women produced comparable amounts of TNF, before and after a single LPS stimulus ($p = 0.4$ and 0.1 , respectively). Both, WBSC from PCOS and normal women, reacted swiftly and equally to a single LPS stimulus ($p = 0.004$ and 0.005 , respectively). No correlation with BMI or W/H ratio was found (results not shown) and plasma cortisol levels in PCOS women (8.1 ± 3.2 mg/dl) and in controls (7.7 ± 2.8 mg/dl) were not significantly different ($p = 0.5$). Serum TNF- α levels in PCOS women (6.73 ± 2.34 pg/ml), were significantly higher ($p = 0.026$) than in controls (4.82 ± 1.15 pg/ml), as shown in Table 2. These TNF concentrations correlated positively ($r = 0.55$, $p = 0.026$) with BMI and negatively ($r = -0.57$, $p = 0.021$) with k_{TT} , as shown in Figures 1A and 1B, respectively.

Table 2 Levels (pg/ml) of TNF- α in WBCS and in serum from women with PCOS

Source of TNF- α	PCOS		NORMAL		P
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	Before LPS (1)	After LPS (2)	Before LPS (3)	After LPS (4)	
WBCS	765 ± 909	4757 ± 2539	1966 ± 2569	5940 ± 1453	1 v/s 3: 0.4 2 v/s 4: 0.1 1 v/s 2: 0.004 3 v/s 4: 0.005
Serum	6.73 ± 2.34		4.82 ± 1.15		0.026

Discussion

TNF- α , a cytokine originally involved in important aspects of the innate immune system, is highly pleiotropic, including effects on the pathophysiology of human reproduction [2]. Since its levels are affected by glucocorticoids [19], interactions among the HPA, the gonadal axis, the innate immune system and stress, may interrupt ovulatory cycles [20-24], a common situation in PCOS women. Consistent with these observations, higher TNF- α serum levels have been detected in this pathology, accompanied in most cases by obesity, insulin resistance [3] and hyperactivity of the HPA axis, expressed as a greater response to human corticotropin-release [29].

As shown in Table 1, in all patients, the levels of 17-OH progesterone, DHEASO₄, total testosterone and prolactin, were less than 2.5 mg/ml, 8 mg/ml, 5.2 nmol/L and 30 ng/ml, respectively, strongly suggesting the absence of other causes for hirsutism and amenorrhea. The biochemical parameters of PCOS women show that, with the exception of increased FAI and androstenedione levels, all other values were within normal ranges. In non-insulin resistant subjects, a fasting insulin level below 18 mIU/ml, less than 60 mIU/ml at 120 minutes, in the oral glucose tolerance test and a k_{ITT} value over 5% are expected. Thus, the observed values in the PCOS women participating in this study suggest a status of resistance to the hormone action, as previously reported (reviewed in ref. [30]).

Higher serum TNF- α levels in PCOS patients (Table 2), and its positive correlation with BMI and negative correlation with k_{ITT} (Figures 1A and 1B, respectively), are in agreement with results from other reports [3]. These

studies also involve subjects with insulin resistance, like in type 2 diabetes mellitus or in android obesity [31-33]. In these patients, the increased levels of this cytokine could also reflect a chronic exposure to high glucocorticoid concentrations, as described in obese subjects [25, 34, 35].

Given the possible role of TNF in the pathogenesis of PCOS, quantification of the capacity of different cell populations to produce this cytokine is important. In PCOS patients there is no available information with regard to the capacity of their cultured blood leukocytes to express TNF- α after an LPS stimulus. Basal or LPS stimulated TNF- α production by circulating macrophage / monocytes can be measured in purified cells or in whole blood (*ex vivo*) short-term cultures (WBSC). This latter system seems to be a better correlate of the *in vivo* situation, for two main reasons. First, by the available techniques for purifying monocytes /macrophages from peripheral blood are lengthy and can mediate non-specific stimuli and even damage these cells [36, 37]. Second, by although lymphocytes (both CD4 and CD8 positive) are able to produce TNF, their presence in the WBSCs does not contribute to the cytokine levels. These cells are stimulated by phorbol diesters or calcium ionophors, while LPS does not induce TNF nor its messenger RNA [8, 9].

When TNF- α levels are measured in humans, blood samples must be withdrawn at controlled times, since circadian variations are known to affect the levels of the cytokine [13, 38]. In this study, 3.00 PM was chosen as a standard time for blood sample collection.

Based on the considerations mentioned above, in this study we used the WBSC experimental approach to ask whether the PCOS syndrome includes an intrinsic altered capacity, basal or LPS stimulated, of cells of the macrophage/monocyte lineage to produce TNF- α . As summarized in Table II, WBSC from PCOS women behaved normally, in their capacity to respond to LPS treatment. Thus, both WBSC from PCOS and normal women produced significantly and equally large amounts of TNF, before or after a single LPS stimulus. No correlation with BMI or W/H ratio was found and plasma cortisol levels in PCOS women (8.1 ± 3.2 mg/dl) and in controls (7.7 ± 2.8 mg/dl) were not significantly different ($p = 0.5$).

Although no significant correlation was found between cortisol levels and basal or over-expressed TNF levels in WBSC, three PCOS patients displaying the highest cortisol levels, were among those showing the lowest response to the

LPS stimulus (results not shown). If these differences have a biological, rather than a statistical meaning, it could be proposed that stress may be handled differently in women with this pathology, as compared to those undergoing normal ovulatory cycles. It could be speculated that the higher levels of cortisol observed in these women could be explained by the increased secretion of the hormone, observed during the post prandial state in abdominal obesity, with inhibition of TNF production [35].

In PCOS patients the presence of specific polymorphic TNF- α genes or their promoters has not been described [4, 39], however the possibility could be entertained that an increase in the cytokine levels plays a role in altering ovary function. Thus, under normal conditions, TNF- α and other cytokines can modulate steroidogenesis in luteal cells during the development of corpus luteum [1, 40, 41]. In a recent report, it has been suggested that TNF- α participates in the pathogenesis of hyperandrogenism in carriers of the -308A variant in the promoter of the cytokine gene [4].

In summary, in this study the *ex vivo* WBSC was chosen as a convenient system to demonstrate that the capacity of blood leukocytes (mainly from the macrophage/monocyte lineage) from PCOS women to produce TNF, under basal and endotoxin stimulated conditions, was comparable with that of normal counterparts. However, given the statistical basis of this interpretation, we can not rule out the possibility that the increased serum levels of the cytokine observed in this reproductive pathology originated in blood leukocytes.

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CONCLUSION

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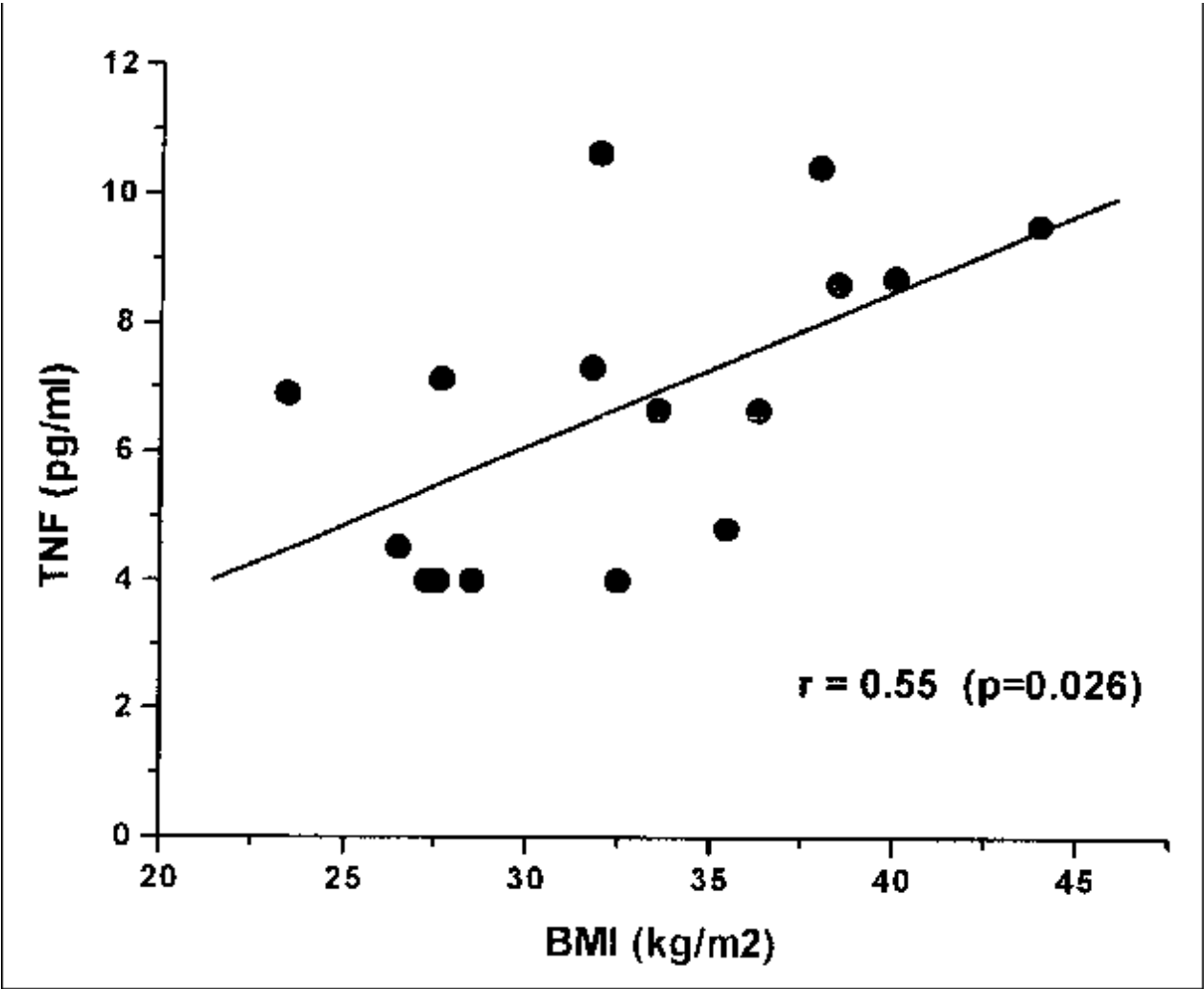
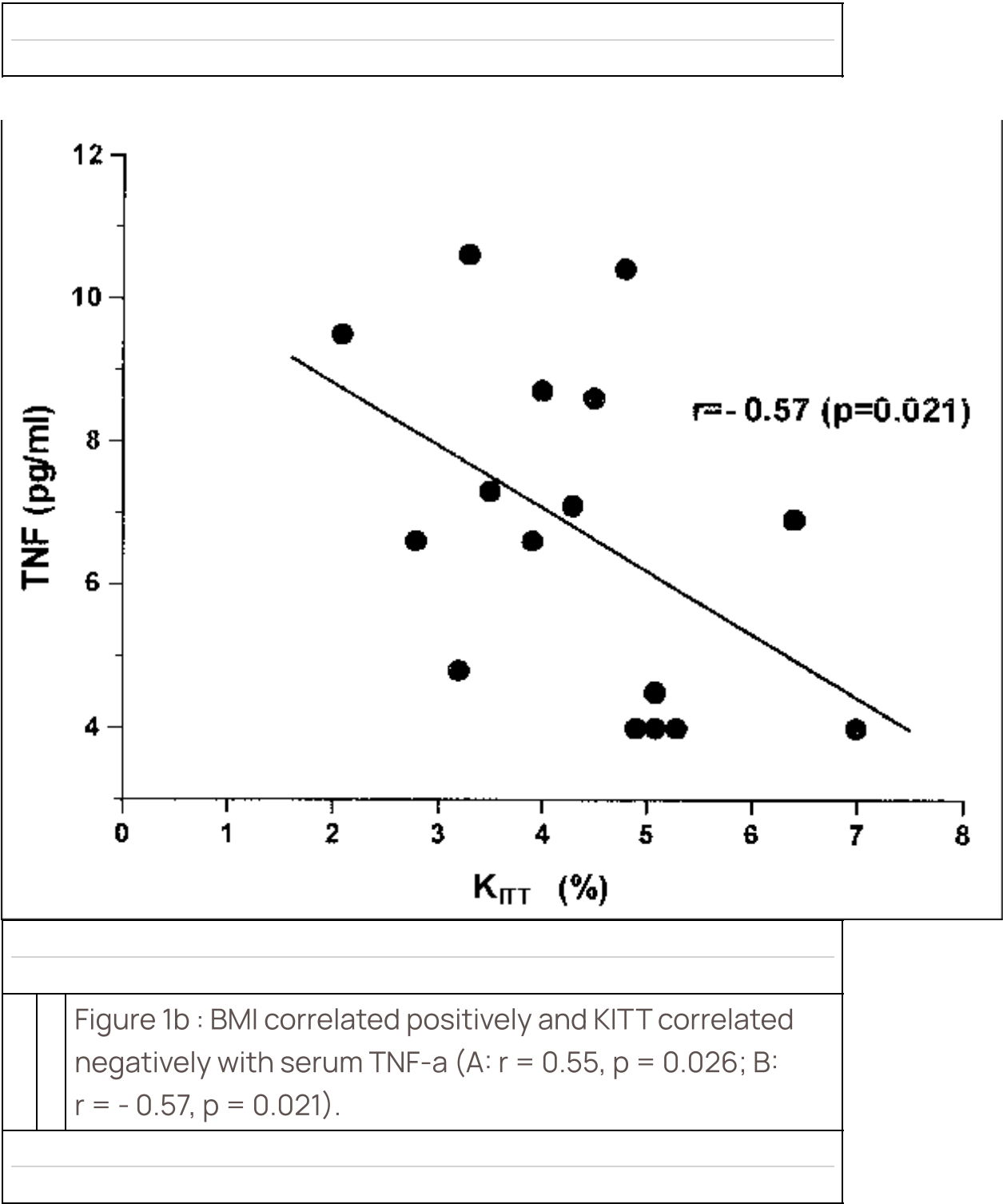


Figure 1a : Correlation between BMI (A) and insulin resistance expressed as KI_{TT} value (B) with serum TNF-a levels in PCOS patients.



Authors

A Veronica Araya, Adam Aguirre, Carmen Romero, Cristian Miranda, Maria Carmen Molina, Arturo Ferreira

Seccion Endocrinologia, Hospital Clinico de la U. de Chile, Santos Dumont 999, Independencia, Santiago, Chile.

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IL-12, IL-18, TGF- β , Plasmodium falciparum

○ In vitro analysis of IGn-gamma and IL-12 production and their effects in ileal Crohn's disease

Stefaan Colpaert, Kathleen Vastraelen, Zhanju Liu, Philippe Maerten, Chong Shen, Freddy Penninckx, Karel Geboes, Paul Rutgeerts, Jan L Ceuppens
IL-12, IFN-g, anti-IL-12/ anti-IFN-g, in vitro, Crohn's disease

○ Differential modulation of IL-2- and IL-4-mediated early activation of normal human B lymphocytes by the caspase inhibitor zVAD-fmk

Shahul Mouhamad, Damien Arnoult, Marie-Thérèse Auffredou, Jérôme Estaquier, Aimé Vazquez
IL-2, IL-4, B lymphocyte, caspase, proliferation, cell cycle entry

○ Prolactin-cytokine network in the defence against *Acanthamoeba castellanii* in murine microglia

Nunzia Benedetto, Claude Auriault
Prolactin, cytokine, *A. castellanii*, MGC.

○ CINC-1 mediates the sympathetic component of inflammatory mechanical hypersensitivity in rats

Berenice B Lorenzetti, Fabiane H Veiga, Claudio A Canetti, Stephen Poole, Fernando Q Cunha, Sergio H Ferreira
Inflammatory hyperalgesia, CINC-1, tumour necrosis factor alpha, interleukin-1b, interleukin-8, sympathetic mediators, prostaglandin E2

○ A CXC chemokine sequence isolated from the rainbow trout *Oncorhynchus mykiss* resembles the closely related interferon-gamma-inducible chemokines CXCL9, CXCL10 and CXCL11

Kerry J Laing, Niels Bols, Christopher J Secombes
Chemokine, rainbow trout, IP10, I-TAC, Mig

○ Meeting report of the "Club Cytokine" (French Society of Immunology) meeting, in Le Croisic, France

Mariette Lisbonne, Hélène Plun-Favreau

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+ 33 (0)1 46 73 06 60

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