

Analysis of soluble angiogenic factors in Crohn's disease: a preliminary study

Inés Dueñas Pousa^a, José Maté Jiménez^a, Xamila Salcedo Mora^a, Maria T. Abreu^b, Ricardo Moreno Otero^a y Javier P. Gisbert^a

^aDepartment of Gastroenterology and Hepatology. Hospital of La Princesa. Autonomous University of Madrid. Spain.

^bDepartment of Medicine. Mount Sinai School of Medicine. New York. USA.

ABSTRACT

AIMS: Soluble angiogenic factors could be altered in patients with Crohn's disease (CD), since inflammation and angiogenesis may play a critical pathogenic role.

METHODS: Serum samples were collected from 30 patients with CD (50% female; median age 44 ± 14 yrs) grouped according to their phenotypic behavior into equal subgroups: inflammatory, fibrostenotic and fistulizing; and 30 healthy controls (50% female; median age 43 ± 14 yrs). Vascular endothelial growth factor (VEGF), placental growth factor (PIGF), angiopoietins (Ang) and receptors (VEGFR1, VEGFR2 and Tie2) in sera were assayed by ELISA.

RESULTS: Serum levels of VEGF (494 ± 247 pg/ml), PIGF (36 ± 11 pg/ml), VEGFR1 (1.9 ± 0.3 ng/ml), Ang2 (6.0 ± 2.3 ng/ml) and Tie2 (36 ± 5 ng/ml) in CD patients were significantly higher than those found in healthy controls (335 ± 118 pg/ml; 23 ± 9 pg/ml; 1.0 ± 0.3 ng/ml; 3.9 ± 2.0 ng/ml; 22 ± 7 ng/ml, respectively). Conversely, CD patients showed significantly lower serum levels of Ang1 than healthy controls (46 ± 11 versus 67 ± 23 pg/ml). In the case of VEGFR2 serum levels, no differences between groups were found. Finally, patients with fibrostenotic CD were characterized by elevated VEGF and Ang2 levels, while patients with an inflammatory phenotype by elevated Tie2 and PIGF levels.

CONCLUSIONS: An activated «pro-angiogenic» profile of angiogenesis soluble markers was observed in CD patients, in comparison with healthy controls. According to the phenotypic behavior, these patients showed differences in serum levels of angiogenic factors.

ANÁLISIS DE LOS FACTORES ANGIOGÉNICOS SOLUBLES EN LA ENFERMEDAD DE CROHN: UN ESTUDIO PRELIMINAR

OBJETIVOS: Los factores angiogénicos solubles podrían estar alterados en los pacientes con enfermedad de Crohn (EC), dado que la inflamación y la angiogénesis pueden desempeñar un papel de carácter crítico en la patogenia de esta enfermedad.

MÉTODOS: Se obtuvieron muestras de suero en 30 pacientes con EC (50% mujeres; edad media, 44 ± 14 años) agrupados subgrupos equivalentes en función de su comportamiento fenotípico: inflamatorio, fibroestenotante y fistulizante, así como en 30 controles sanos (50% mujeres; edad media, 43 ± 14 años). Mediante análisis de inmunoabsorción ligada a enzimas (Elisa, *enzyme-linked immunoadsorbent assay*) se evaluaron en el suero el factor de crecimiento endotelial vascular (VEGF, *vascular endothelial growth factor*), el factor de crecimiento placentario (PIGF, *placental growth factor*), las angiopoyetinas (Ang) y los receptores VEGFR1, VEGFR2 y Tie2.

RESULTADOS: Las concentraciones séricas de VEGF (494 ± 247 pg/ml), PIGF (36 ± 11 pg/ml), VEGFR1 (1.9 ± 0.3 ng/ml), Ang2 (6.0 ± 2.3 ng/ml) y Tie2 (36 ± 5 ng/ml) en los pacientes con EC fueron significativamente mayores que las observadas en los controles sanos (335 ± 118 pg/ml; 23 ± 9 pg/ml; 1.0 ± 0.3 ng/ml; 3.9 ± 2.0 ng/ml; 22 ± 7 ng/ml, respectivamente). Por el contrario, los pacientes con EC presentaron concentraciones séricas significativamente inferiores de Ang1, en comparación con los controles sanos (46 ± 11 y 67 ± 23 pg/ml, respectivamente). En el caso de las concentraciones séricas de VEGFR2, no se observaron diferencias entre los 2 grupos. Finalmente, los pacientes con EC fibroestenotante se caracterizaron por una elevación de las concentraciones de VEGF y Ang2, mientras aquellos que tenían el fenotipo inflamatorio presentaron concentraciones elevadas de Tie2 y PIGF.

CONCLUSIONES: En los pacientes con EC se detectó un perfil «proangiogénico» activado en lo relativo a los marcadores solubles de la angiogénesis, en comparación con lo observado en los controles sanos. Según el comportamiento fenotípico, estos pacientes presentaron diferencias en las concentraciones séricas de los factores angiogénicos.

Correspondence: J.P. Gisbert.

Playa de Mojácar, 29. Urb. Bonanza. 28669 Boadilla del Monte (Madrid). España.

E-mail: gisbert@meditex.es

Recibido el 21-3-2007; aceptado para su publicación el 5-6-2007.

INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disorder that involves different sites along the length of the gastrointestinal tract. The well known transmural and scarring character of this inflammatory bowel disease can explain the common local complications, such as deep sinus tracts and fistulas, mesenteric abscesses, and obstruction¹.

Although remarkable advances have occurred in recent years in the diagnosis and therapy of CD, the factors which lead to differences in the clinical evolution of this disorder among diverse patients are still unknown¹. The finding of pathophysiologically relevant markers reflecting disease progression would be helpful in order to predict the evolution of CD patients or to optimize the treatment for each individualized patient. Diverse serum mediators have been analyzed in order to propose them as biological markers, but a general agreement does not exist in this field².

Angiogenesis, or the formation of new vessels from pre-existing ones, is a natural process that occurs in the organism in association with both healthy and pathologic conditions³. This complex process, which involves endothelial cell proliferation, migration, and adhesion, matrix remodeling, recruitment of pericytes and stabilization of neovessels, is triggered through mechanisms requiring the participation of proinflammatory cytokines and growth factors^{3,4}. Inflammatory mediators can, either directly or indirectly, promote angiogenesis. Angiogenesis, in turn, contributes to pathological inflammation by permitting the recruitment of inflammatory cells; therefore, inflammation and angiogenesis are linked processes⁵. Neoangiogenesis is tightly regulated by a balance of pro- and anti-angiogenic factors, which are expressed in response to external stimulatory or inhibitory agents⁶. Under pathological conditions, an excess of pro-angiogenic factors occurs, leading to an 'angiogenic switch', which initiates the pathological formation of new vessels⁶.

In the case of chronic inflammatory diseases, angiogenesis is initiated as a defence mechanism aimed at preventing tissue ischemia caused by inflammatory infiltrates and at facilitating the access of immune cells to the site of injury. However, the newly formed vascular network is structurally and functionally abnormal and may also contribute to tissue damage⁷. Derived from several studies, blocking agents against angiogenic factors have been proposed as potential candidates for diverse chronic inflammatory diseases treatment⁸. The study of the relationship between vascular development and inflammatory bowel diseases could be helpful to elucidate the pathogenesis of this chronic inflammatory disease^{7,9}.

Among the major mediators implicated in the angiogenic process are the members of vascular endothelial growth factor (VEGF) family (VEGF and placental growth factor, PlGF), the angiopoietins (Ang), both Ang-1 and Ang-2, and their respective tyrosine-kinase receptors: VEGF receptors (VEGFR1 and VEGFR2) and Ang1 and Ang2 receptor, Tie2^{3,6,7}.

Little data exist regarding the role of soluble angiogenic factors in the pathogenesis of CD. Regarding VEGF, previous results are controversial, possibly due to the diverse population of the patients studied and the differences in the laboratory's techniques used⁹⁻¹³, which are probably not suitable for comparing them. In the case of Ang2 and Tie2, one study has measured their serum levels in patients with active and inactive CD and found significant differences in respect to the healthy controls¹⁴. Therefore, as angiopoietins may play a critical role in adult angiogenesis related with the inflammation, they should be further investigated in CD.

In this preliminary study, we analyzed whether serum levels of angiogenic factors are increased in patients with CD as compared with healthy controls. We further evaluated whether patients with distinct CD clinical phenotypes express different amounts of angiogenic factors in order to find a possible marker, which could predict the evolution of the EC patients. Thus, serum levels of several angiogenic factors (VEGF, PlGF, and their receptors, as well as the angiopoietins –Ang1 and Ang2–, and the receptor Tie2) were studied in CD patients, which were stratified into inflammatory, fibrostenotic and fistulizing sub-types. Finally, we attempted to elucidate the possible separate or combined role of all these soluble factors on CD.

METHODS

Patients and controls

Thirty patients (50% female, ranging in age from 23 to 72 yrs; mean age, 44 ± 14 yrs) with a diagnosis of CD and Crohn's Disease Activity Index (CDAI) ≤ 150 were studied. Diagnosis of CD was established by standard clinical, radiological, histological, and endoscopic criteria¹⁵. Patients were matched for age and sex with thirty healthy controls (50% female, ranging in age from 25 to 71; mean age, 43 ± 14 yrs). Using the Vienna classification, CD patients were classified according to their clinical phenotypic behavior into 3 equal sub-groups: inflammatory, fibrostenotic and fistulizing. Signed informed consent was obtained from each participant under a protocol approved by the Ethical Committee on Clinical Investigation of our hospital.

All patients and controls were Caucasian. Patients receiving biologic therapies, or those presenting with cancer, coronary disorders, malignant hypertension and pregnant women were excluded from this study. Serum levels of standard laboratory parameters including hemoglobin, leukocyte count, platelet count, albumin, erythrocyte sedimentation rate, C-reactive protein, fibrinogen and orosomucoid were measured by routine determination.

Determination of angiogenic soluble factors

Serum levels of VEGF, PlGF, VEGFR1, VEGFR2, Ang1, Ang2 and Tie2 were determined both in CD patients and healthy controls. Peripheral blood samples were allowed to clot for 30 min at -4°C temperature before centrifugation for 10 min at 2500-3000 rpm. Serum was stored at -80°C . The angiogenic soluble factors levels were measured by enzyme-linked immunosorbent assays (ELISA) (Human Immunoassay Quantikine, R&D Systems, Minneapolis, USA). Their concentrations were determined by the standard curve obtained when using the standards supplied with the kit according to the manufacturer's instructions.

Statistical analysis

For quantitative variables, mean and standard deviation were calculated. Student T-test was used to carry out the comparison between serum levels of angiogenic factors in CD patients and healthy controls. Statistical analysis in order to evaluate the differences of angiogenic soluble factors concentrations depending on the behavior of the disease was per-

formed by the analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons. The relationship among the serum levels of the diverse angiogenic factors was analyzed by a linear regression model. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Demographic and clinical characteristics of both CD patients and healthy controls are summarized in table I. Of the 30 evaluated patients, 50% were women, 50% smokers, 10% had extraintestinal manifestations and 43% had ileocolonic disease. The mean of the evolution of the disease was 10.2 ± 6 years. With regard to the phenotypic behavior of the disease, 10 patients were included in each sub-group: 10 inflammatory, 10 fibrostenotic and 10 fistulizing. 76.7% of EC patients received maintenance therapy: 20.0% aminosalicylates and 53.3% immunomodulators, including azathioprine, mercaptopurine and methotrexate.

In CD patients, most of the soluble angiogenic factors showed significantly higher serum levels than in healthy controls (VEGF: 494 ± 247 vs. 335 ± 118 pg/ml, $p < 0.01$; PlGF: 36 ± 11 vs. 23 ± 9 pg/ml, $p < 0.001$; VEGFR1: 1.9 ± 0.3 vs. 1.0 ± 0.3 ng/ml, $p < 0.001$; Ang2: 6.0 ± 2.3 vs. 3.9 ± 2.0 ng/ml, $p < 0.001$; Tie2: 36 ± 5 vs. 22 ± 7 ng/ml, $p < 0.001$) (fig. 1). Conversely, lower Ang1 serum levels were observed in CD patients than in healthy controls (46 ± 11 vs. 67 ± 23 ng/ml, $p < 0.001$) (fig. 1). However, no difference existed in VEGFR2 serum levels between CD patients (10.2 ± 2.0 ng/ml) and healthy controls (10.4 ± 3.2 ng/ml).

Results were also compared according to the behavior of CD. In patients with fibrostenotic CD, a tendency towards increased VEGF (610 ± 237 pg/ml) and Ang2 (7.2 ± 2.6 ng/ml) levels compared to those with inflammatory (439 ± 213 pg/ml and 5.2 ± 1.2 ng/ml, respectively) or fistulizing (432 ± 268 pg/ml and 5.7 ± 2.4 ng/ml, respectively) behavior was observed (fig. 2). On the other hand, the patients with inflammatory phenotype showed slightly elevated PlGF (38 ± 18 pg/ml), Ang1 (49 ± 12 ng/ml), and Tie2 (38 ± 5 ng/ml) levels compared with fibrostenotic (35 ± 5 pg/ml, 46 ± 9 ng/ml and 36 ± 5 ng/ml), and fistulizing (35 ± 6 pg/ml, 43 ± 11 ng/ml and 35 ± 4 ng/ml) behavior (fig. 2). However, these trends did not reach statistical significance, probably due to the limited sample size. Noteworthy is the fact that there were statistically significant elevated Ang1 serum levels in CD patients with perianal fistulas in comparison with those with internal fistulas (51 ± 11 vs. 35 ± 3 ng/ml, $p < 0.05$). Significant differences of the levels of the angiogenic factors were not observed when EC patients were classified according to the fact of presenting stenosis (yes/no) or fistulas (yes/no), independently.

When the CD patients were compared according to their disease location pattern, differences did not reach statistical significance. In colonic CD, VEGF (336 ± 151 pg/ml) and Ang2 (5.4 ± 1.7 ng/ml) serum levels were lower than in ileal (590 ± 268 pg/ml; 7.1 ± 2.8 ng/ml, respectively) or ileocolonic CD (524 ± 247 pg/ml; 5.7 ± 2.1 ng/ml, respectively).

TABLE 1. Demographic and clinical characteristics of Crohn's disease (CD) patients and healthy controls

	Crohn's disease	Healthy control
Number of patients	30	30
Age, mean (SD)	44 (14)	43 (14)
Gender		
Female	15	15
Male	15	15
CD localization		
Ileal	9	-
Ileocolonic	13	-
Colonic	8	-
CD behavior		
Inflammatory	10	-
Fibrostenotic	10	-
Fistulizing	10	-
Perianal	5	-
Internal	5	-

SD: standard deviation.

The EC patients who received maintenance therapy (76.7%) showed an increase of the levels of VEGF and a decrease of the VEGFR2 levels as compared with those who did not take such medication (557 ± 243 vs. 287 ± 113 , $p < 0.001$; 9.8 ± 2.0 vs. 11.3 ± 1.2 , $p < 0.05$, respectively). Additionally, significant altered levels of VEGF and Ang1 in EC patients who took immunomodulators regarding those who did not take azathioprine, mercaptopurine or methotrexate as maintenance therapy were observed (VEGF: 583 ± 236 vs. 391 ± 224 , $p < 0.05$; Ang1: 42 ± 10 vs. 51 ± 10 , respectively).

The correlation between pairs of these angiogenic factors was also studied. VEGF correlated positively with Ang2 ($r = 0.42$, $p = 0.001$). VEGFR1, as well as its ligand, PlGF, correlated positively with Ang2 ($r = 0.42$, $p = 0.001$; $r = 0.30$, $p = 0.019$, respectively) and Tie2 ($r = 0.76$, $p < 0.001$; $r = 0.53$, $p < 0.001$, respectively). However, they showed negative correlation with Ang1 ($r = -0.45$, $p < 0.001$; $r = -0.30$, $p = 0.021$, respectively). Correlations were also observed between receptors and its ligands. Thus, VEGFR1 correlated with PlGF ($r = 0.58$, $p < 0.001$), and Tie2 with Ang2 ($r = 0.30$, $p = 0.021$, positively) and with Ang1 ($r = -0.49$, $p < 0.001$, negatively).

Moreover, CD patients showed a statistically significant correlation between serum VEGF levels and leukocyte count ($r = 0.42$, $p = 0.03$). Finally, serum levels of both VEGFR1 and VEGFR2 were correlated with orosomucoid ($r = 0.49$, $p = 0.021$; $r = 0.44$, $p = 0.042$, respectively).

DISCUSSION

In the current preliminary study, CD patients presented a pro-angiogenic profile reflected by elevated serum levels of two angiogenic systems consisting of ligands and their receptors, VEGF-PlGF/VEGFR1 and Ang1-Ang2/Tie2. This supports the hypothesis that an alteration in the neoangiogenic process participates in the pathogenesis of CD. Most of our knowledge about the VEGF-

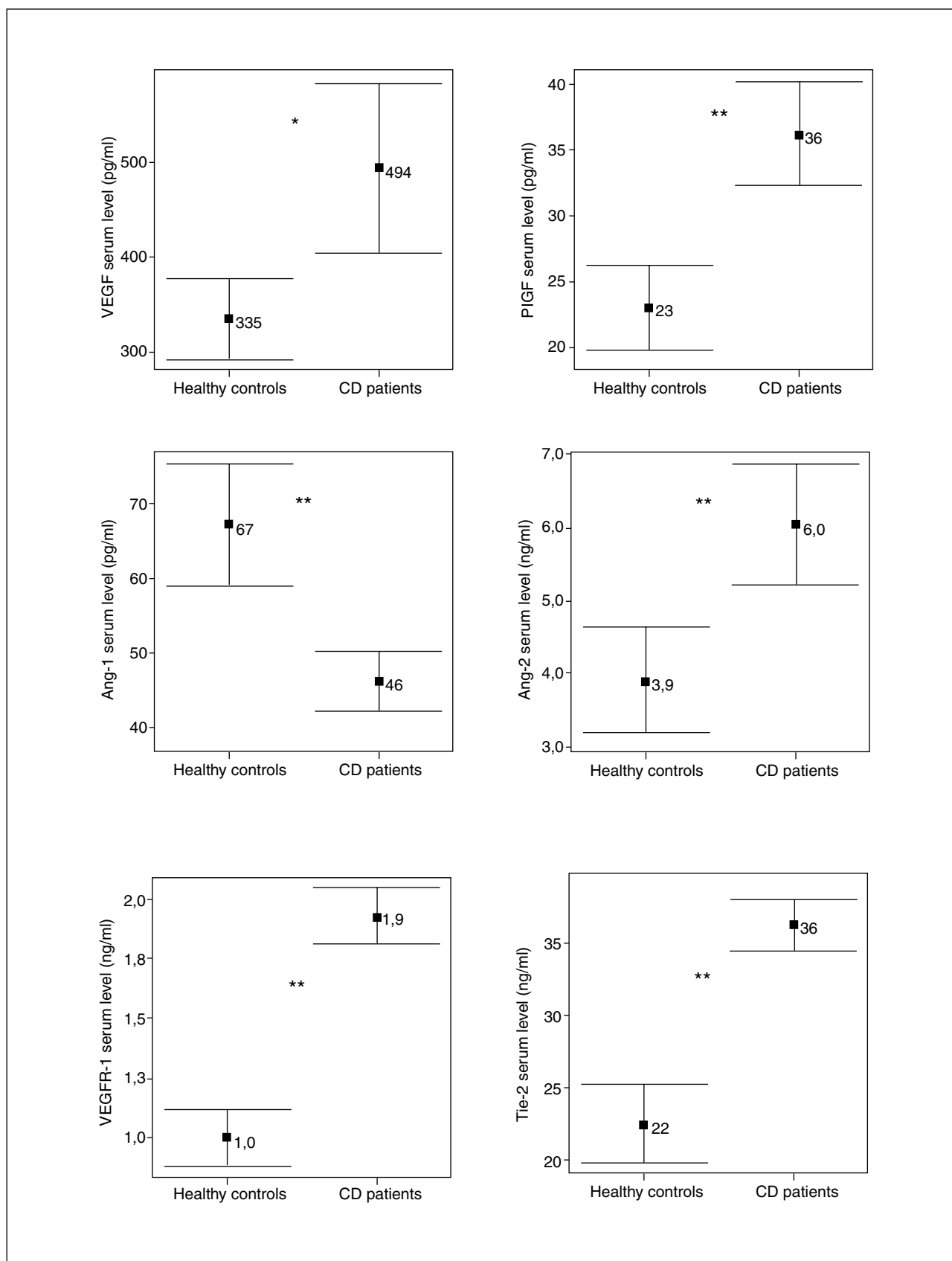


Fig. 1. Comparison between angiogenic soluble factors in healthy controls versus Crohn's disease (CD) patients. * $p < 0.01$, ** $p < 0.001$. Error bars plots are given with means and standard deviations.

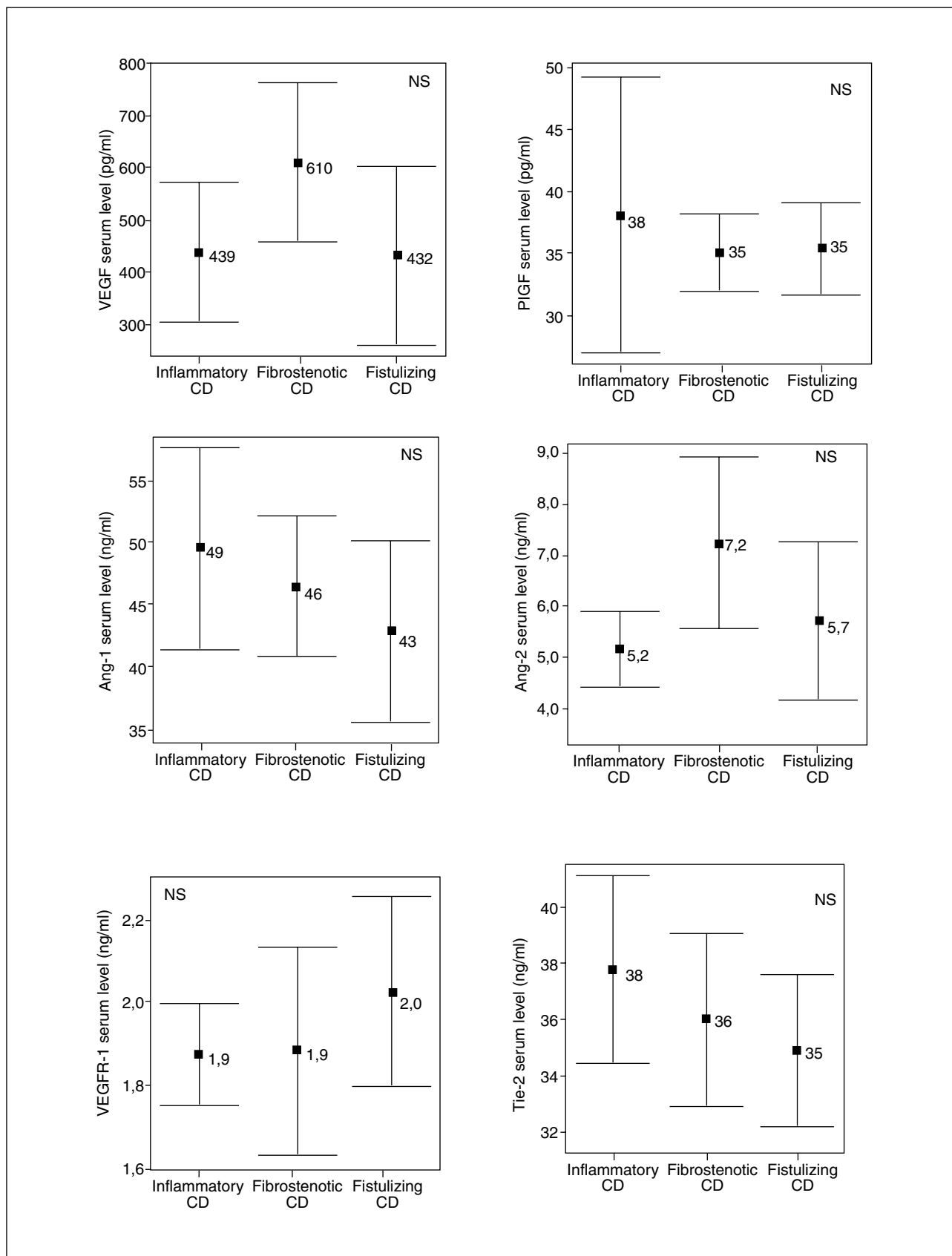


Fig. 2. Comparison of the angiogenic soluble factors depending on the behavior of the Crohn's disease (CD). NS: non-statistically significant differences for all comparison. Error bars plots are given with means and standard deviations.

PIGF/ VEGFR1 system arises from recent oncology studies in which the PIGF has shown to play an important role in the angiogenic switch in pathological angiogenesis by interacting with VEGFR1 and synergizing with VEGF¹⁶.

In theory, we would expect that the soluble form of the VEGFR1 would function as a «decoy» during the development of blood vessels, and prevent VEGF from binding to its signaling receptors. However, PIGF, which is produced and released in abundant amounts by activated endothelial cells, could displace VEGF from VEGFR1, thereby favoring the fraction of VEGF available to bind to and activate VEGFR2¹⁷. The same may occur with the extracellular domain of Tie2 that is detectable in serum and its two ligands, Ang1 and Ang2. One of them may displace the other to interact with the binding ligand site of the soluble receptor.

In our study, CD patients presented statistically significant higher serum levels of VEGF, PIGF and VEGFR1 than healthy controls. Few reports exist that focus on the differences of angiogenic serum factor levels between CD patients and healthy controls. Our findings are consistent with previous reports, that were carried out in children and young adults with CD^{11,18}, in which an increased number of VEGF and VEGFR1-positive blood vessels in colonic mucosa were seen¹⁰. Conversely, other authors observed either no differences between these two groups^{9,12,19} or even lower serum levels of VEGF in CD patients^{13,20}. In the case of the Ang1-Ang2/Tie2 system, information on its pathophysiological role in CD is even scarcer. There is only one study that has observed elevated Ang2 and Tie2 serum levels in active CD patients compared with healthy controls¹⁴ and, to the best of our knowledge, the present study is the first one to examine Ang1 serum levels and the relationships among diverse angiogenic factors in CD patients.

Both Tie2 and Ang2 levels are increased in CD patients in remission, allowing the development of new vessels, together with the system VEGF-PIGF/VEGFR1. By contrast, the Ang1 levels in these patients were lower than in healthy controls, suggesting that there is a decoy of the constitutive stabilizing effects of this angiopoietin on new blood vessels. This suggests that an abnormal and pathological angiogenesis in the bowel wall exists, and thereby further studies would be necessary to evaluate if these new vessels are irregularly shaped, tortuous, and lack the normal hierarchical arrangement, as in the case of tumor blood vessels²¹. These results are consistent with the knowledge that angiogenesis requires blockage of Ang1-mediated signaling and hence Ang2 tends to be upregulated by stimulus associated with angiogenesis including VEGF²².

Perhaps the most interesting observations in this study are the differences of soluble angiogenic factors when the patients were classified according to CD behavior. However, these differences were not statistically significant, possibly due to the limited number of patients investigated. The fibrostenotic behavior of CD was characterized by elevated VEGF and Ang2 levels, which could favor

the formation of new blood vessels, supplying nutrients and oxygen to the new tissue. On the other hand, an increase of Tie2 and PIGF levels in the inflammatory pattern, both of them previously related with inflammation⁶ and activation of monocytes²³, was observed. Moreover, the differences in Ang1 serum levels of CD patients with internal or perianal fistulas could reflect the local disturbed equilibrium between the systems of angiopoietins. These findings could be helpful to elucidate the evolution of each CD patient and thereby to adapt the follow-up or the therapeutic strategy.

The intake of maintenance therapy, especially immunomodulators, may have a significant influence on the levels of VEGF, VEGFR2 and Ang1 in EC patients. However, in our opinion these results are not conclusive due to the inequality of the group compared.

Observed correlations between some pairs of angiogenic factors reflect a possible interplay between these systems of angiogenic mediators. Thus, it could be more appropriate to evaluate several angiogenic mediators rather than only one. Moreover, the obtained correlation among the members of the system Ang1-Ang2/Tie2 reflects, as already described, the necessary balance between resting and activated vascular endothelium⁵.

To conclude, angiogenesis in CD patients appears to play a role in CD reflected by abnormal levels of angiogenic mediators. Moreover, differences seem to be present in patients according to their clinical phenotypic behavior. Wider and prospective studies are necessary to determine if these angiogenic soluble markers could be useful to predict the evolution of patients with CD.

ACKNOWLEDGEMENTS

This study was supported by a grant (to ID Pousa) from Schering Plough and by grants (to JP Gisbert) from Instituto de Salud Carlos III (C03/02 and PI050109).

REFERENCES

1. Stange EF, Travis SP, Vermeire S, Beglinger C, Kupcinkas L, Geboes K, et al. European evidence based consensus on the diagnosis and management of Crohn's disease: definitions and diagnosis. *Gut*. 2006;55 Suppl 1:1-15.
2. Gisbert JP, González-Lama Y, Mate J. Role of biological markers in inflammatory bowel disease. *Gastroenterol Hepatol*. 2007;30:117-29.
3. Carmeliet P. Angiogenesis in health and disease. *Nat Med*. 2003;9:653-60.
4. Pousa ID, Gisbert JP, Mate J. Vascular development in inflammatory bowel disease. *Gastroenterol Hepatol*. 2006;29:414-21.
5. Beat A, Imhof MA-L. Angiogenesis and inflammation face off. *Nat Med*. 2006;12:171-2.
6. Salcedo X, Medina J, Sanz-Cameno P, García-Buey L, Martín-Vilchez S, Moreno-Otero R. Review article: angiogenesis soluble factors as liver disease markers. *Aliment Pharmacol Ther*. 2005;22:23-30.
7. Medina J, Arroyo AG, Sánchez-Madrid F, Moreno-Otero R. Angiogenesis in chronic inflammatory liver disease. *Hepatology*. 2004;39:1185-95.
8. Ranieri G, Ria R, Roccaro AM, Vacca A, Ribatti D. Development of vasculature targeting strategies for the treatment of chronic inflammatory diseases. *Curr Drug Targets Inflamm Allergy*. 2005;4:13-22.

9. Danese S, Sans M, De la Motte C, Graziani C, West G, Phillips MH, et al. Angiogenesis as a novel component of inflammatory bowel disease pathogenesis. *Gastroenterology*. 2006;130:2060-73.
10. Konno S, Iizuka M, Yukawa M, Sasaki K, Sato A, Horie Y, et al. Altered expression of angiogenic factors in the VEGF-Ets-1 cascades in inflammatory bowel disease. *J Gastroenterol*. 2004; 39:931-9.
11. Kader HA, Tchernev VT, Satyaraj E, Lejnine S, Kotler G, Kingsmore SF, et al. Protein microarray analysis of disease activity in pediatric inflammatory bowel disease demonstrates elevated serum PLGF, IL-7, TGF-beta1, and IL-12p40 levels in Crohn's disease and ulcerative colitis patients in remission versus active disease. *Am J Gastroenterol*. 2005;100:414-23.
12. Kanazawa S, Tsunoda T, Onuma E, Majima T, Kagiya M, Kikuchi K. VEGF, basic-FGF, and TGF-beta in Crohn's disease and ulcerative colitis: a novel mechanism of chronic intestinal inflammation. *Am J Gastroenterol*. 2001;96:822-8.
13. Magro F, Araujo F, Pereira P, Meireles E, Diniz-Ribeiro M, Velosom FT. Soluble selectins, sICAM, sVCAM, and angiogenic proteins in different activity groups of patients with inflammatory bowel disease. *Dig Dis Sci*. 2004;49:1265-74.
14. Koutroubakis IE, Xidakis C, Karmiris K, Sfiridaki A, Kandidaki E, Kouroumalis EA. Potential role of soluble angiopoietin-2 and Tie-2 in patients with inflammatory bowel disease. *Eur J Clin Invest*. 2006;36:127-32.
15. Lennard-Jones JE. Classification of inflammatory bowel disease. *Scand J Gastroenterol*. 1989;170 Suppl:2-6.
16. Autiero M, Waltenberger J, Communi D, Kranz A, Moons L, Lambrechts D, et al. Role of PIGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. *Nat Med*. 2003;9:936-43.
17. Autiero M, Luttun A, Tjwa M, Carmeliet P. Placental growth factor and its receptor, vascular endothelial growth factor receptor-1: novel targets for stimulation of ischemic tissue revascularization and inhibition of angiogenic and inflammatory disorders. *J Thromb Haemost*. 2003;1:1356-70.
18. Bousvaros A, Leichtner A, Zurakowski D, Kwon J, Law T, Keough K, et al. Elevated serum vascular endothelial growth factor in children and young adults with Crohn's disease. *Dig Dis Sci*. 1999;44:424-30.
19. Griga T, Tromm A, Spranger J, May B. Increased serum levels of vascular endothelial growth factor in patients with inflammatory bowel disease. *Scand J Gastroenterol*. 1998;33:504-8.
20. Kapsoritakis A, Sfiridaki A, Maltezos E, Simopoulos K, Giatromanolaki A, Sivridis E, et al. Vascular endothelial growth factor in inflammatory bowel disease. *Int J Colorectal Dis*. 2003; 18:418-22.
21. Baluk P, Hashizume H, McDonald DM. Cellular abnormalities of blood vessels as targets in cancer. *Curr Opin Genet Dev*. 2005;15:102-11.
22. Hangai M, Murata T, Miyawaki N, Spee C, Lim JI, He S, et al. Angiopoietin-1 upregulation by vascular endothelial growth factor in human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci*. 2001;42:1617-25.
23. Perelman N, Selvaraj SK, Batra S, Luck LR, Erdreich-Epstein A, Coates TD, et al. Placenta growth factor activates monocytes and correlates with sickle cell disease severity. *Blood*. 2003; 102:1506-14.