Altered thiol pattern in plasma of subjects affected by rheumatoid arthritis

D. Giustarini*, S. Lorenzini[1*], R. Rossi, D. Chindamo[1], P. Di Simplicio, R. Marcolongo[1]

Department of Neuroscience, Pharmacology Unit and [1]Department of Clinical Medicine and Immunological Sciences, Rheumatology Unit, University of Siena, Italy.

Abstract

Objective

Rheumatoid arthritis (RA) is a chronic inflammatory disease which involves the synovial membrane of multiple diarthroidal joints causing damage to cartilage and bones. The damage process seems to be related to an overproduction of oxygen reactive species inducing an oxidative perturbation. Since sulfhydryl groups are primary antioxidant factors, we were interested in investigating the balance of plasma sulfhydryl/disulfides in patients with active RA compared to healthy control subjects.

Methods

Twenty-one patients with RA and 15 age-matched controls were studied. Plasmatic sulfhydryl groups and their disulfide form concentrations were measured by spectrophotometry or HPLC.

Results

RA patients showed significantly lower levels of plasma protein sulfhydryls and cysteinyl-glycine compared to healthy controls ($p < 0.001$). Conversely, cystine and homocystine, and protein-bound cysteine and homocysteine were significantly increased ($p < 0.005$ in disulfides forms and $p < 0.05$ in protein mixed disulfides forms). There was a significant correlation between some clinical data (ESR, number of tender/swollen joints) and some of the parameters studied.

Conclusion

The results of this study indicate a biochemical disturbance of plasma sulfhydryl/disulfides balance in patients with RA compared to controls with an increase in some oxidised forms (disulfides and protein mixed disulfides) and a decrease in free thiols. The increase in total homocysteine, correlated to the higher risk of cardiovascular diseases in RA patients, is associated with higher levels of the oxidised forms, disulfides and protein-thiol mixed disulfides.

Key words

Rheumatoid arthritis, redox thiol status, homocysteine, protein mixed disulfides, oxidative stress.

*These authors contributed equally to the work.

Daniela Giustarini, PhD; Sauro Lorenzini, PhD; Ranieri Rossi, PhD; Daniela Chindamo, MD; Paolo Di Simplicio, BSc; and Roberto Marcolongo, MD.

Please address correspondence to: Dr. Sauro Lorenzini, Department of Clinical Medicine and Immunological Sciences, Rheumatology Unit, Viale Bracci 1, University of Siena, Italy.
E-mail: lorenzini@unisi.it

Received on July 2, 2004; accepted in revised form on November 30, 2004.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease of multi-factorial origin which involves the synovial membrane of diarthroidal joints leading to erosive damage to cartilage and bones (1, 2). Inflammatory cells, including granulocytes, play a relevant role in the development of the joint damage and, in particular, in cartilage destruction, through the production of reactive oxygen species (ROS) and the release of lysosomial enzymes, that promote oxidative stress and enhance inflammation (3, 4).

Human cells have developed a strong antioxidant defence against these pro-oxidant reactions (5). In particular, they possess an enzymatic pattern (e.g. superoxide dismutase, glutathione peroxidase, catalase, glutathione reductase, glucose 6-phosphates dehydrogenase) and non-enzymatic antioxidant molecules, thiols (mainly glutathione) ascorbic acid, α-tocopherol and the urate (6). An important chemical barrier against oxidative stress damage is the redox equilibrium of sulfhydryl/disulfides, through which low molecular weight thiols can be reversibly oxidised to disulfides and/or protein mixed disulfides (reacting with cysteine residues in proteins) in response to an oxidative status. Consequently, blood/ plasma thiol levels can be considered as useful markers of inflammation status, as their balance is strongly influenced by the oxidative perturbation generated by this condition.

The association between rheumatoid arthritis and an alteration in plasma sulfhydryl content has been investigated in the past: total thiol concentration was lower in the serum/plasma of subjects affected by RA (7-9). Furthermore, it has been suggested that the beneficial therapeutic effects of D-penicillamine (a thiol-containing molecule) (10, 11) and Myocrisin (a gold (I) compound containing a complexed thiol) (12) in the treatment of RA may be due to their influence on the thiol system. Various molecules are known to possess sulfhydryl groups in plasma, with different properties in terms of concentrations and reactivity. The most abundant fraction is in albumin, that has a cysteine (Cys) residue in position 34 (13). Its normal sulfhydryl concentration is around 0.7 mol/albumin molecule (14): this non-integer number occurs because some thiols are blocked by the formation of a disulfide bond between the albumin thiol and low molecular weight thiols, specifically Cys, cysteinylglycine (CysGly), homocysteine (Hcys) and glutathione (GSH), physiologically present in plasma (15).

Since plasma, as all extracellular fluids, is normally exposed to oxidant agents (also in healthy subjects) these low molecular weight thiols are in equilibrium with their oxidised forms. The oxidation of the sulfhydryl group in these molecules brings about the formation of corresponding disulfides: cystine (CySS), cystinylglycine (CyGlySS), homocystine (HcySS), glutathione disulfide (GSSG) and/or low molecular weight mixed disulfides and/or protein mixed disulfides.

The aim of this study was to determine the plasma sulfhydryl/disulfides balance in patients with active RA by analysing all thiol redox species that had been potentially modified as a result of the disease.

Materials and methods

Study subjects

The study group was comprised of 21 consenting patients of a mean age of 61 years (14 women and 7 men) with active RA, according to the criteria of the ACR (2) with a mean duration of 11.5 years of disease. Recruitment criteria for study entry included tender and swollen joint count > 6 and erythrocyte sedimentation rate (ESR) > 28 mm/h (Table I) (16).

All patients had positive serum IgM rheumatoid factor. None of the patients had any alterations in renal function (evaluated measuring the serum levels of creatinine and urea) nor in hepatic function (evaluated measuring the activity of γGT and transaminases). Thirteen patients received disease modifying antirheumatic drugs (DMARDs) (methotrexate 7.5 mg/week, hydroxychloroquine 400 mg/day, parenteral gold 100 mg/week leflunomide 20 mg/day), 15 were taking nonsteroid anti-inflammatory drugs and 14 were taking

Table I. Description of the study population.*

Parameters	RA group	Control group
N° of subjects	21	15
Age	61.1 ± 11.9	57.4 ± 7.6
Sex (F:M)	14:7	11:4
Duration of arthritis (yrs.)	11.5 ± 6.3	-
N° of tender joints	24.5 ± 6.1	-
N° of swollen joints	23.7 ± 6.1	-
ESR, mm/h	51.9 ± 21.8	15.3 ± 6.9
Creatinine, mg/dl	0.81 ± 0.20	0.85 ± 0.25
γGT, UI/l	25.7 ± 11.9	16.8 ± 6.11
Therapy (%)	Steroids 66.7%	-
	NSAIDS 71.4%	-
	DMARD 61.9%	-

* Except where otherwise indicated, values are expressed as mean ± SD

oral corticosteroids (prednisolone < 10 mg/day). None of the patients were given folate and vitamin B_{12} supplements.

Fifteen healthy sex-age matched controls (11 women and 4 men, mean age 57.4 years) were also studied. All the healthy subjects enrolled in this study were asymptomatic and none of them had any abnormality on physical examination nor in routine laboratory blood tests.

Patients were examined at the beginning of our study for their selection and the choice of therapy. Successively, after 2 months, measurements of clinical and biochemical parameters have been performed. Patients have been visited and blood samples have been drawn for both clinical data (Table I) and thiols/disulfides analyses. Clinical parameters have been measured within 1-2 hours from the blood collection accordingly to routine laboratory analyses protocols. All subjects enrolled gave their informed consent to the study; the study was approved by the local ethical committee.

Biochemical analyses

HPLC-grade reagents were purchased from BDH (Poole, Dorset, England). A 4.6 x 250 mm reversed-phase HPLC column Sephasil C18 was purchased from Pharmacia (Uppsala, Sweden). Monobromobimane (mBrB) was obtained from Calbiochem (La Jolla, CA, USA). All other reagents of analytical grade were from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

Blood samples were drawn from both groups from the antecubital vein, collected in plastic tubes containing K_3EDTA and immediately centrifuged for 20s at 10000g. Plasma was rapidly withdrawn and derivatised for thiol, disulfides and protein mixed disulfides measurement. Our procedure was similar to that indicated by Mansoor *et al.* (15) with slight modifications (17). The remaining sample was kept at 0°C until the protein sulfhydryl determination (carried out within 1h).

Protein SH group (PSH) determination was carried out spectrophotometrically by their conjugation with 5,5'-dithiobis-(2-nitrobenzoic acid) (18). Measures were carried out using a Jasco V-550 spectrophotometer.

For low molecular weight thiols determination, plasma was immediately deproteinised by acidification with 6% (w/v) TCA (final concentration). After centrifugation (10000g, 2 min) supernatants were incubated with 1 mM final concentration monobromobimane (mBBr) at neutral pH obtained with solid $NaHCO_3$ (19). After 15 min incubation at room temperature in the dark, samples were acidified with an adequate amount of 37% (v/v) HCl and analysed by HPLC. In order to obtain a linear standard curve for thiols at low concentrations, we preincubated mBBr before samples derivatization with 20 μM N-acetylcysteine (final concentration) for 15 min.

Disulfides were measured on plasma samples pre-treated with 5 mM N-ethylmaleimide (NEM, final concentration). After a few minutes samples were acidified with 6% (w/v, final concentration) TCA and centrifuged at 10000g, 2 min. The excess NEM in the supernatants was extracted with dichloromethane (0.2 ml sample+2 ml dichloromethane), then samples were treated with 1 mM dithiotreitol (DTT) (final concentration) to reduce disulfide bond, restoring pH to a neutral condition by solid $NaHCO_3$. After a 20 min at room temperature samples were incubated with an excess of mBBr and treated for HPLC analysis as indicated above.

Protein mixed disulfides (RSSP) were determined in the same samples utilised for disulfide determinations. Pellets obtained after TCA acidification were washed twice with 1.5% (w/v) TCA in order to remove traces of NEM, low molecular weight thiols and disulfides. Then pellets were solubilized in H_2O containing 2 mM DTT at neutral pH with solid $NaHCO_3$. After 20 min samples were deproteinised with the appropriate amount of TCA and the thiols, released from the disulfide bond by DTT reduction, were conjugated with an excess of mBBr at neutral pH and treated for HPLC analysis as described above.

Samples were injected into a Pharmacia Sephasil column C_{18}, 250 x 4 mm. Solvent A was 0.25% (v/v) acetic acid, pH 3.09 and solvent B was methanol. The elution profile was as follows: 0-8 min, 20% B; 8-15 min, 20-40% B; and 15-25 min, 40-100% B. The HPLC apparatus was a Hewlett Packard Series 1100 instrument. Derivatised thiols were analysed by fluorescence detection (excitation, 380 nm; emission, 480 nm) and quantified using external standards of similarly derivatised Cys, Cys-Gly, Hcys, GSH, with a detection limit of 100 nM.

The albumin concentration was determined by standard clinical chemistry procedures.

Statistical analysis

The studied populations were analysed by Kolmogorov-Sminorv test and showed a Gaussian distribution. Nevertheless, due to the small number of sam-

Table II. Plasma levels of thiols, disulfides, and protein mixed disulfides in subjects affected with RA and in control subjects. Values are expressed as mean ± SD.

Group	Thiols (μM)					Disulfides (μM)				Mixed disulfides (μM)			
	SH albumin	Cys	CysGly	Hcys	GSH	CySS	CyGlySS	HcySS	GSSG	Cy-SSP	CyGly-SSP	Hcy-SSP	GSSP
AR	316 ± 49.8**	8.33 ± 2.26	1.26 ± 0.43**	0.223 ± 0.138	2.13 ± 1.12	74.0 ± 11.4**	4.67 ± 1.28	2.05 ± 1.13**	1.10 ± 0.54	171 ± 31*	10.3 ± 3.06	11.4 ± 5.35*	1.73 ± 1.07
Control	422 ± 52	9.32 ± 1.14	1.97 ± 0.41	0.21 ± 0.11	1.96 ± 0.66	62.7 ± 9.49	5.33 ± 1.18	1.06 ± 0.52	1.18 ± 0.47	147 ± 24	11.3 ± 1.76	7.52 ± 2.84	1.73 ± 0.66

*p < 0.05, RA group vs control subjects (Mann-Whitney test).
**p < 0.005, RA group vs control subjects (Mann-Whitney test).
Cys: cysteine; CysGly: cysteinylglycine; Hcys: homocysteine; GSH: glutathione; CySS: cystine; CyGlySS: cystinylglycine; HcySS: homocystine; GSSG: glutathione disulfide; CySSP: protein mixed disulfide with cysteine; CyGlySSP: protein mixed disulfide with cysteinylglycine; HcySSP: protein mixed disulfide with homocysteine; GSSP: protein mixed disulfide with GSH.

ples we have preferred to apply non parametric tests. Mann Whitney test has been used to compare plasma thiol status in control and RA groups. Correlation with clinical parameters in each study group were quantified by the Spearman correlation test.

Results

Plasma thiols/disulfides

Plasma thiols, both low molecular weight (LMWSH) and protein thiols, and also their disulfide forms were determined in control subjects and in RA patients (Table II). Protein thiol concentration, mainly consisting in albumin SH groups, in patients was significantly lower compared to the control group ($p<0.005$). Analysis of normalised PSH values with albumin concentration (HSA) confirmed a significant decrease in the RA group (8.40 ± 1.23 vs 10.0 ± 1.30 nmoles/mg protein, $p < 0.005$, data not shown), suggesting that this result is not due to a simple depletion of HSA in patients but to a real decrease in protein sulfhydryl groups content (8, 20).

Our data confirmed that, with exception of glutathione, LMWSHs in plasma are much less abundant compared to disulfides and protein-thiols mixed disulfides (15) and indicated that their concentration range is 0.1–10 μM with Cys>CysGly>GSH>Hcys. The amount of each low molecular weight thiol was very similar in the plasma of control subjects and RA patients; only CysGly showed a significant decrease in the RA group (1.26 vs 1.97 μM, $p < 0.005$).

Plasma disulfides were mainly CySS, while the other species (namely GSSG, HcySS, CyGlySS) were present at lower levels; a significant increase in cystine and homocystine concentration in RA patients was observed ($p<0.005$).

The profile of plasma levels of RSSP matched that of disulfides, as cysteine-protein mixed disulfide (CySSP) level was much more abundant, and the other species were lower by 1 to 2 orders of magnitude. Also in this case, patients showed a significant increase in CySSP and homocysteine-protein mixed disulfide (HcySSP) plasma levels ($p < 0.05$).

As a whole, our data indicate a significant prevalence of the oxidised forms in RA patients. This aspect has been described in details in Figure 1. In par-

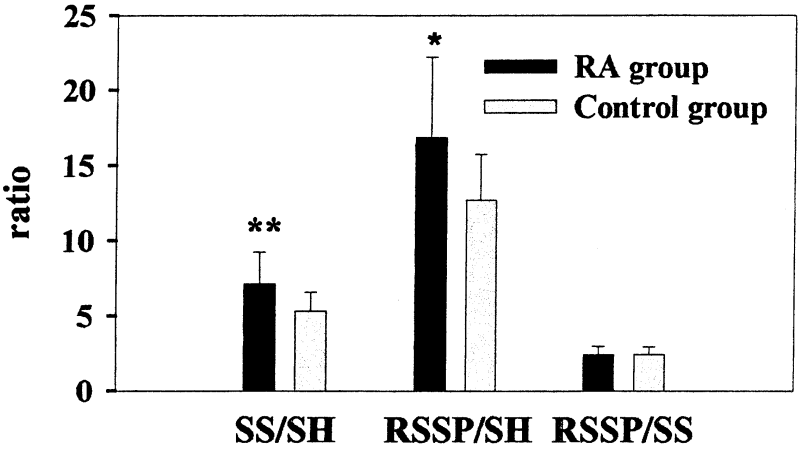

Fig. 1. Ratio value between disulfides and low molecular weight thiols, between protein mixed disulfides and low molecular weight thiols and between protein mixed disulfides and disulfides concentration in the plasma of the RA and control groups.
*p<0.05, RA group vs control subjects (Mann-Whitney test).
**p<0.005, RA group vs control subjects (Mann-Whitney test).

ticular, the calculated ratio between disulfides and thiols was significantly higher in RA patients ($p < 0.005$), indicating that the pathology is associated with a pro-oxidant status. Moreover, this aspect was confirmed by the calculated ratio between protein-mixed disulfides and LMWSH in the RA group, with a significantly higher value compared to control subjects ($p < 0.05$). No significant differences were found between the two groups in the RSSP and disulfides ratio.

Correlation between clinical data and different redox forms of thiols

Measured parameters in plasma were then correlated with some clinical and hematological data of Table I. As showed in Figure 2, in the RA group, ESR values were inversely related with the concentration of plasma CyGlySS ($r^2 = 0.201$; $p < 0.05$). Interestingly, one significant correlation was found between the number of tender joints and cysteine-protein mixed disulfides concentration, $r^2 = 0.269$; $p < 0.05$ (Fig. 3). The same result was obtained replacing the number of tender joints with the number of swollen joints ($r^2 = 0.242$, $p < 0.05$, not shown).

Protein sulfhydryls

Since albumin posses one free cysteine, a significant percentage of which is involved in mixed disulfides formation with low molecular weight thiols, theoretically, in every subject the sum of protein mixed disulfide plus free protein sulfhydryl groups concentration should be equal to the albumin concentration. In Figure 4 we have reported the percentage of each fraction (i.e. PSH and RSSP in the studied groups) compared to albumin concentration (corresponding to 100%). Our data show that in healthy subjects most of the albumin Cys34 is found as free thiol, whereas about 30% is oxidised to mixed disulfide with low molecular weight thiols. There is a low percentage of thiols which was not detected in our measurements (PSX) but was found in the difference between albu-

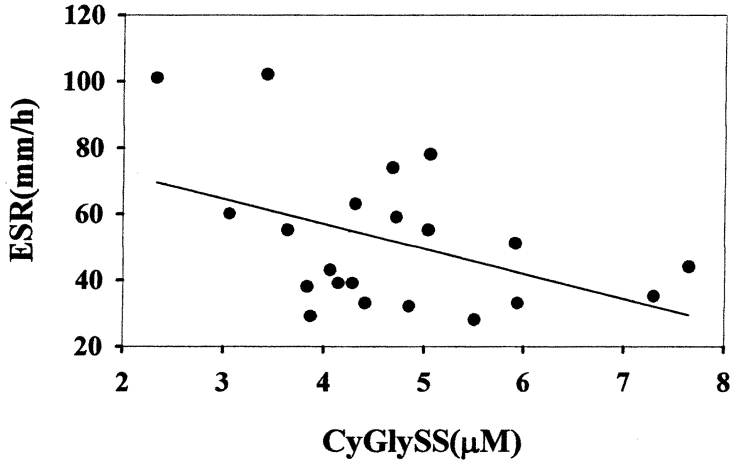

Fig. 2. Correlation between the concentration of plasma cysteinylglycine disulfide and ESR values in RA patients. $r^2 = 0.201$; $p < 0.05$, Spearman correlation test.

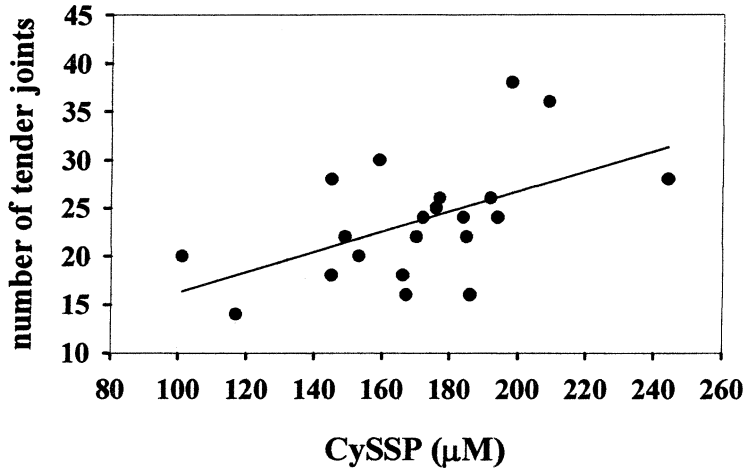

Fig. 3. Correlation between the concentration of plasma cysteine-protein mixed disulfides bound and the number of tender joints in RA patients. $r^2 = 0.269$ $p < 0.05$, Pearson's correlation test.

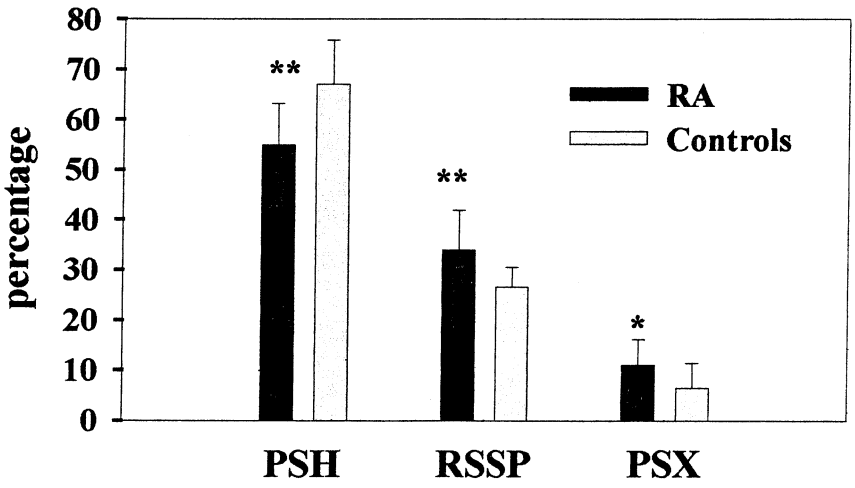

Fig. 4. Percentage of albumin measured as PSH (free thiol), RSSP (protein mixed disulfides with low molecular weight thiols) or PSX. PSX, constitutes the amount of sulfhydryl groups not measured but calculated as the difference between the total amount of albumin (100%) and the sum of PSH plus RSSP.
*p < 0.05, RA group vs control subjects (Mann-Whitney test).
**p<0.005, RA group vs control subjects (Mann-Whitney test).

min and PSH+RSSP concentrations. In RA patients protein sulfhydryl group fraction was significantly lower than in healthy subjects ($p<0.005$) and an increase in RSSP fraction was found ($p < 0.005$). Interestingly, also the amount of PSXs was significantly higher in RA patients ($p<0.05$).

Discussion

To our knowledge, this is the first paper assessing the levels of LMWSH and their disufides related forms in plasma of RA patients in comparison with healthy subjects.

It is known that plasma LMWSH (except glutathione) are more abundant in the oxidised forms, particularly as protein mixed disulfides (15) and our data in both control and RA group (Table II) confirmed this. Cysteine, cystine and protein-cysteine mixed disulfide are the most concentrated species found as free thiols or disulfide.

Reduced thiols showed a similar distribution in age and sex matched RA patients: a significant decrease was observed only for CysGly levels. As a whole we observed a slight decrease of total reduced thiols compared to controls (12.0 μM vs 13.5 μM) (Table II). The decrease in CysGly content in RA patients could be due to the fact that this molecule could be particularly sensitive to an oxidative insult being its sulfhydryl group more reactive than other thiols (21,22). Since we did not find a correspondent increase in its disulfide form, we may speculate the formation of undetectable forms after strong oxidation of the SH group (sulphenic, sulphinic, sulphonic acid). This could in part explain the inverse correlation we found between CyGlySS levels and ESR values in RA subjects (Fig. 2); it is possible that the CysGly sulfhydryl group, by virtue of its higher reactivity, undergoes first stronger oxidative modifications during inflammatory processes related with RA pathogenesis.

Cisteine and homocysteine were significantly higher in the plasma of RA patients, both as low molecular weight disulfides and as protein mixed disulfide with albumin. As cysteine is a by-product of homocysteine degradation, it is possible that its increase is due to Hcys overproduction. Nevertheless, since cysteine oxidised forms are quantitatively more abundant than the corresponding homocysteine forms, we also cannot exclude alternative patterns for cysteine overproduction (e.g, release from the cellular environment). Data of Table II indicate a significant increase in total homocysteine levels in RA group (15.8 μM vs 9.8 μM in control group, $p<0.005$). Our results indicate that this raise is mainly due to the disulfide and the protein bound forms. This increase in homocysteine levels was not provoked by renal insufficiency or nephropathy because all the patients enrolled in this study had a serum level of creatinine and urea within the reference range.

It has also been observed that methotrexate administration could interfere with homocysteine levels since it is a folate antagonist, and folate is a cofactor required for methionine resynthesis from homocysteine but some data exist indicating the absence of a direct correlation (23,24). Additionally, only 48% of our patients received low doses of methotrexate (7.5mg/week) and by comparing the homocysteine levels in the group taking methotrexate with the group that did not, we did not observe significant differences for each redox form (not shown). Moreover, increased plasma levels of total homocysteine in rheumatoid arthritis patients have been previously measured (25-27). Therefore we believe that the increase of both HcySS and HcySSP, observed in RA subjects, was not significantly influenced by the therapy. Useful information could derive from these data in that it is known that hyperhomocysteinemia is an important risk factor for vascular diseases, thrombotic events and stroke and, what is more, that cardiovascular disorders are the first cause

of death in RA patients (25, 28-30).

The rise of protein mixed disulfides with low molecular weight thiols levels in AR patients was responsible, at least in part, for the parallel decrease in protein sulfhydryl content indicated by our measurements (see Table II and Fig. 4) [and also previously observed (8, 20)]. Although it has been observed that albumin concentrations can decrease in patients with RA (31), our normalised data regarding HSA confirmed this trend (data not shown). Nevertheless data from Figure 4 seem indicate also a further sulfhydryls oxidation (for example, sulfenic, sulfinic, or sulfonic acid, undetectable with our method), due to a heavier oxidative burden during RA pathogenesis.

Therefore, on the whole our data indicate the occurrence of a pro-oxidant status in RA subjects, as deduced by data of Figure 1. Both oxidised forms (disulfides and protein mixed disulfides) in RA were significantly more concentrated than in control group. This could be partially explained by previous data indicating a higher plasma copper content in RA patients compared to controls, as this metal is involved in radical producing reactions (21, 32-34).

The increase in RSSP content and in particular, in CySSP could have a prognostic value in RA occurrence. In fact, we observed that plasma CySSP levels were directly related with the number of tender (Fig. 3) and swollen joints (data not shown). Interestingly it should be considered that many antirheumatic drugs, such as penicillamine (10, 11, 35), gold-thiol complexes, (e.g. aurothiomalate and aurothioglucose) (12), esonarimod (KE-298) (36), sulfasalazine (37) influence the thiol redox status or are thiols themselves. It derives from this that a modulation of thiol redox status may have a role both in the pathogenesis of the disease or in the therapeutic benefit of some drugs. It has also been observed that the dietary intake of certain antioxidant micronutrients may be protective against the development of RA (38, 39), thus reinforcing the hypothesis that a modulation of redox balance may be involved.

In conclusion, our data indicate that RA patients are characterised by decreased levels of thiols and increased concentration of their disulfide forms, with some of these parameters closely related to articular damage and ESR. Homocystine, cystine and corresponding protein-thiol mixed disulfides could be used as useful markers for the evaluation of the activity of the pathology. Therefore the analysis of these parameters could represent an additional information with respect to other existing measures of disease activity (e.g. CRP) for suggesting some indications about the more pertinent therapeutical approach. The possibility that an alteration in the plasma thiols/disulfides balance is a factor that concurs to the development of the disease, and not only a secondary phenomenon derived from increased ROS production by activated neutrophils is an interesting hypothesis that requires further studies.

References

1. HARRIS ED: Rheumatoid arthritis: pathophysiology and implications for therapy. *N Engl J Med* 1990; 322: 1227-89.
2. ARNETT FC, EDWORTHY SM, BLOCH DA *et al.*: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31: 315-24.
3. GREENWALD RA: Oxygen radicals, inflammation, and arthritis: pathophysiological considerations and implications for treatment. *Semin Arthritis Rheum* 1991; 20: 219-40.
4. SCHILLER J, FUCHS B, ARNHOLD J, ARNOLD K: Contribution of reactive oxygen species to cartilage degradation in rheumatic diseases: molecular pathways, diagnosis and potential therapeutic strategies. *Curr Med Chem* 2003; 10: 2123-45.
5. SIES H: Strategies of antioxidant defense. *Eur J Biochem* 1993; 215: 213-9.
6. SIES H: Oxidative stress: from basic research to clinical application. *Am J Med* 1991; 91: 31S-8S.
7. HAATAJA M, KALLIOMAKI JL: Serum sulphydryl groups in rheumathoid arthritis. *Z Rheumathol* 1977; 36: 73-6.
8. BANFORD JC, BROWN DH, HAZELTON RA, MCNEIL CJ, SMITH WE, STURROCK RD: Altered thiol status in patients with rheumatoid arthritis. *Rheumatol Int* 1982; 2: 107-11.
9. DI SIMPLICIO P: An improved method of determination of SH group concentration and reactivity in plasma of patients with various clinical disorders and in rats after administration of indomethacin or carbon tetrachloride. *Pharmacol Res Commun* 1983; 15: 805-14.
10. MUNTHE E, JELLUM E, AASETH J: Some aspects of the mechanism of penicillamine in rheumatoid arthritis. *Scand J Rheumatol* 1979; 28: 6-12.
11. HOWARD-LOCK HE, LOCK CJ, MEWA A, KEAN WF: D-penicillamine: chemistry and clinical use in rheumatic disease. *Semin Arthritis Rheum* 1986; 15: 261-81.
12. BROWN DH, SMITH WE: The chemistry of the gold drugs used in the treatment of rheumatoid arthritis. *Chem Soc Rev* 1980; 9: 217-40.
13. CARTER D, HO J: Structure of serum albumin. *Adv Protein Chem* 1994; 45: 153-203.
14. SENGUPTA S, CHEN H, TOGAWA T *et al.*: Albumin thiolate anion is an intermediate in the formation of albumin-S-S-homocysteine. *J Biol Chem* 2001; 276: 30111-7.
15. MANSOOR MA, SVARDAL AM, UELAND P: Determination of the *in vivo* redox status of cysteine, cysteinylglycine, homocysteine, and glutathione in human plasma. *Anal Biochem* 1992; 200: 218-29.
16. RITCHIE DM, BOYLE JA, MCINNES JM *et al.*: Clinical studies with on articular index for the assessment of joint tenderness in patients with rheumatoid arthritis. *Q J Med* 1968; 37: 393-406.
17. GIUSTARINI D, CAMPOCCIA G, FANETTI G *et al.*: Minor thiols cysteine and cysteinylglycine regulate the competition between glutathione and protein SH groups in human platelets subjected to oxidative stress. *Arch Biochem Biophys* 2000; 380: 1-10.
18. ELLMAN GL: Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959; 82: 70-7.
19. NEWTON GL, FAHEY RC: Determination of biothiols by bromobimane labeling and high-performance liquid chromatography. *Methods Enzymol* 1995; 251: 148-66.
20. KOSTER JF, BIEMOND P, SWAAK AJG: Intracellular and extracellular sulphydryl levels in rheumatoid arthritis. *Ann Rheum Dis* 1986; 45: 44-6.
21. SENGUPTA S, WEHBE C, MAJORS AK, KETTERER ME, DI BELLO PM, JACOBSEN DW: Relative roles of albumin and celluloplasmin in the formation of homocystine, homocysteine-cysteine-mixed disulfide, and cystine in circulation. *J Biol Chem* 2001; 276: 46896-904.
22. DAUBEUF S, LEROY P, PAOLICCHI A *et al.*: Enhanced resistance of HeLa cells to cisplatin by overexpression of gamma-glutamyltransferase. *Biochem Pharmacol* 2002; 64: 207-16.
23. VAN EDE AE, LAAN RF, BLOM HJ, DE ABREU RA, VAN DE PUTTE LB: Methotrexate in rheumatoid arthritis: an update with focus on mechanisms involved in toxicity. *Semin Arthritis Rheum* 1998; 27: 277-92.
24. HUEMER M, FODINGER M, HUEMER C *et al.*: Hyperhomocysteinemia in children with juvenile idiopathic arthritis is not influenced by methotrexate treatment and folic acid supplementation: a pilot study. *Clin Exp Rheumatol* 2003; 21: 249-55.
25. HERNANZ A, PLAZA A, MARTIN-MOLA E, DE MIGUEL E: Increased plasma levels of homocysteine and other thiol compounds in rheumatoid arthritis women. *Clin Biochem* 1999; 32: 65-70.
26. ROUBENOFF R, DELLARIPA P, NADEAU MR *et al.*: Abnormal homocysteine metabolism in rheumatoid arthritis. *Arthritis Rheum* 1997; 40: 718-22.

27. SERIOLO B, FASCIOLO D, SULLI A, CUTOLO M: Homocysteine and antiphospholipid antibodies in rheumatoid arthritis patients: relationships with thrombotic events. *Clin Exp Rheumatol* 2001; 19: 561-4.
28. WILLIAMS RH, MAGGIORE JA, REYNOLDS RD, HELGASON CM: Novel approach for the determination of the redox status of homocysteine and other aminothiols in plasma with ischemic stroke. *Clin Chem* 2001; 47: 1031-9.
29. CLARKE R, DALY L, ROBINSON K *et al.*: Hyperhomocysteinemia: an independent risk factor for vascular disease. *N Engl J Med* 1991; 334: 1149-55.
30. MYLLYKANGAS-LUOSUJARVI R, AHO K, KAUTUIANEN H, ISOMAKI H: Cardiovascular mortality in women with rheumatoid arthritis. *J Rheumatol* 1995; 22: 1065-7.
31. ROPES M, PERLMANN GE, KAUFMAN D, BAUER W: Electrophoretic distribution of proteins in plasma in rheumatoid arthritis. *J Clin Invest* 1954; 33: 311-8.
32. TUNCER S, KAMANLI A, AKCIL E, KAVAS GO, SECKIN B, ATAY MB: Trace element and magnesium levels and superoxide dismutase activity in rheumatoid arthritis. *Biol Trace Elem Res* 1999; 68: 137-42.
33. ZOLI A, ALTOMONTE L, CARICCHIO R *et al.*: Serum zinc and copper in active rheumatoid arthritis: correlation with interleukin 1 beta and tumour necrosis factor alpha. *Clin Rheumatol* 1998; 17: 378-82.
34. RAFTER GW: Plasma thiols, copper and rheumatoid arthritis. *Med Hypotheses* 1994; 43: 59-61.
35. HARADA S, SUGIYAMA E, TAKI H *et al.*: D-penicillamine cooperates with copper sulfate to enhance the surface expression of functional Fas antigen in rheumatoid synovial fibroblasts via the generation of hydrogen peroxide. *Clin Exp Rheumatol* 2002; 20: 469-76.
36. NOGUCHI T, ONODERA A, TOMISAWA K, YAMASHITA M, TAKESHITA K, YOKOMORI S: Synthesis and antirheumatic activity of the metabolites of esonarimod. *Bioorg Med Chem* 2002; 10: 2713-21.
37. GUPTA V, JANI JP, JACOBS S *et al.*: Activity of melphalan in combination with the glutathione transferase inhibitor sulfasalazine. *Cancer Chemother Pharmacol* 1995; 36: 13-9.
38. CERHAN JR, SAAG KG, MERLINO LA, MIKULS TR, CRISWELL LA: Antioxidant micronutrients and risk of rheumatoid arthritis in a cohort of older women. *Am J Epidemiol* 2003; 157: 345-54.
39. HELGELAND M, SVENDSEN E, FORRE O, HAUGEN M: Dietary intake and serum concentrations of antioxidants in children with juvenile arthritis. *Clin Exp Rheumatol* 2000; 18: 637-41.