Plasma endogenous enkephalin levels in early systemic sclerosis: clinical and laboratory associations

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ABSTRACT

Objective. *Met- and leu-enkephalins are endogenous opioid neuropeptides with potent analgesic, vasoactive, immunomodulatory and anti-apoptotic properties. We hypothesised that clinical or immunological variables of early systemic sclerosis (SSc) might be correlated to plasma enkephalin levels.*

Methods. *Plasma samples were collected at study entry of the Genetics versus Environment in Scleroderma Outcomes Study (GENISOS) cohort (early SSc, n=116). Plasma met-enkephalin and leu-enkephalin levels (ug/ml) were measured by high performance liquid chromatography (HPLC) and correlated to clinical and laboratory parameters in the GENISOS database. Statistical analyses were performed by nonparametric Wilcoxon rank sum tests and Pearson correlation coefficients.*

Results. *Significantly lower plasma met-enkephalin levels were associated with anti-topoisomerase-I seropositivity (6+8.3 vs. 14.9+22.8 ug/ml, p=0.02). Plasma leu-enkephalin levels were significantly higher in SSc patients with digital pulp loss (95.6+130 vs. 64.9+101 ug/ml, p=0.02). Lower mean plasma met-enkephalin levels and inversely higher leu-enkephalin levels were noted in SSc patients with Raynaud's phenomena (p=NS).*

Conclusion. *The associations of plasma enkephalin levels to immunologic or clinical pathologies may underscore their vasogenic or fibrogenic significance and potential as therapeutic targets in early SSc.*

Introduction

Systemic sclerosis (SSc, scleroderma) is a multisystemic autoimmune disease characterised by prominent widespread small vessel vasculopathy and endothelial damage with resultant fibrosis of skin and organs. Recurring episodes of vasoconstriction with tissue ischemia, then reperfusion generates well known clinical signs of ischemic vascular injury. These include Raynaud's phenomenon, capillary damage, digital ulcers, pitting scars and digital pulp loss. Activation of myofibroblasts contributes to obliterative narrowing of arterioles and fibrosis of skin and organs (Reviewed in 1, 2).

Recent studies in SSc have focused on the impact of vasoactive mediators and disease specific autoantibodies in the initiation and persistence of vasoconstriction, fibrogenic morbidity, luminal narrowing and clinical progression (1, 2). Elevated levels of vasogenic mediators and receptor activation such as endothelin-1 and endothelin A and B receptors and norepinephrine and alpha adrenergic receptors have potent vasoactive influences on the endothelium in SSc (3, 4). SSc specific anti-topoisomerase I antibody (TOPO-1) has been associated with the promotion of fibrogenic processes (4) and subsequent fibrotic contractures in the digits (5).

The enkephalins are endogenous opioid neuropeptides with potent analgesic, vasoactive, immunomodulatory and anti-apoptotic properties in the central nervous system and peripheral tissues (Reviewed in 6, 7). They are primary ligands for μ- and δ-opioid receptors and have increased expression in the endothelium of injured tissue. Enkephalins are recognised as prominent vasodilators of small and medium-sized arteries, especially in animal models of brain injury (8). Thus, we hypothesised that plasma endogenous enkephalin levels might be associated with vasogenic or subsequent fibrogenic changes in SSc vasculopathy. In this study, we analysed plasma enkephalin levels for significant associations with clinical or serologic variables that potentially impact vascular or fibrotic pathogenic processes in patients with early SSc.

Material and methods

The research protocol was approved by Institutional Review Boards and

General Clinical Research Centers of participating universities and written informed consent was obtained from all subjects before study entry. The GENISOS samples were patients with early SSc who met American College of Rheumatology diagnostic criteria within 5 years of diagnosis, or onset of the first symptom other than Raynaud's phenomenon, detailed elsewhere (9, 10). Patients were diagnosed with systemic sclerosis (SSc) if they met the ACR proposed classification criteria (10). Assignment of diffuse cutaneous SSc was further based on the proximal distribution of skin involvement, that is, thickened skin involvement detected above the elbows or knees, exclusive of involvement of the face (11). Nailfold capillaroscopy was not performed as part of the GENISOS study. The patients were enrolled throughout the year. The catchment area of the three participating university centres (UTMB, UTH-HSC, and UTHSC-SA) roughly encompasses a 500-mile area in Southeastern Texas, with a latitude range of 29.3–29.75 degrees. The overall daily temperatures range from 14–33°C (12). All clinical visits were performed in climate controlled buildings, usually 23–26°C for patient comfort. Plasma samples (n=116) were aliquoted and stored at -80°C until HPLC analysis for enkephalin determinations was performed by blinded investigators. For HPLC analysis, thawed filtered samples were subjected to reverse phase HPLC with coulometric electrochemical detection (ESA product 5010A) for met-enkephalin and leu-enkephalin (13). For statistical analyses, nonparametric Wilcoxon's rank sum test was used to compare met- and leu-enkephalin levels based on the presence or absence of clinical or serologic manifestations. Pearson correlation coefficients were then computed to examine the association between plasma levels of met-enkephalin and leu-enkephalin and continuous demographic, clinical or serologic variables such as age, skin score, manifestations of digital lesions, modified health assessment questionnaire, short form-36 physical component summary (PCS) or mental component summary (MCS) scores and tobacco use from the GENISOS database at study entry. Patients were assessed for disease manifestations by analysing study patients in whom variables on the clinical manifestations form (CMF) were marked as "present today". Samples with determinations for met- and leu-enkephalin levels and responses for the available database variables were analysed for this study. Multiple linear regression analyses were conducted to investigate persistently significant differences after controlling the effects of age, race and gender. Data are presented as mean ± standard deviation unless otherwise specified. A p-value <0.05 was considered significant. Power analysis was performed to determine if our sample size was adequate to attain significance by multiple regression analysis (14).

Table I. Association of plasma enkephalin levels to clinical variables.

Scleroderma Clinical Profile	Variable (n.)		Met-Enk, (ug/ml)	p-value	Leu-Enk, (ug/ml)	p-value
Serum TOPO-1	Yes	(23)	6.1 ± 8.3	0.02	80.1 ± 99.1	0.35
	No	(93)	14.9 ± 22.8		74.1 ± 116.0	
Loss of digital pulp	Yes	(37)	8.5 ± 13.4	0.10	97.0 ± 134	0.04
	No	(79)	14.8 ± 23		65.1 ± 100	
Raynaud's phenomenon	Yes	(109)	12.8 ± 21.4	0.07	78.2 ± 114.8	0.11
	No	(7)	16.6 ± 5.9		25.9 ± 29.2	
Tendon friction rubs	Yes	(10)	3.5 ± 3.2	0.06	128.3 ± 236.4	0.54
	No	(106)	14.0 ± 21.7		70.0 ± 92.0	
Myositis	Yes	(5)	9.9 ± 14.6	0.74	14.3 ± 11.1	<0.05
	No	(111)	13.2 ± 21.2		77.7 ± 113	
Telangiectasias	Yes	(27)	12.1 ± 16.4	0.47	44.6 ± 58.9	0.09
	No	(89)	13.3 ± 22.1		85.0 ± 123.2	
Diffuse cutaneous SSc	Yes	(65)	12.5 ± 16.4	1.00	84.4 ± 128.1	0.72
Limited cutaneous SSc	Yes	(51)	13.8 ± 26.0		64.6 ± 90.6	

Association of plasma enkephalin levels and clinical parameters in early SSc. Plasma samples and clinical data were obtained from the baseline study visit of the GENISOS database. Plasma Met-enkephalin and leu-enkephalin levels are expressed as mean values ± standard deviation. The presence (yes, "present today") or absence (no) of the parameter at the time of the study visit is noted and the (number) represents the number of patients in the database. The p-value demonstrates the level of significance from univariate analysis. The number of samples with the present variable ("yes") or absence ("no") for a clinical or laboratory variable are shown in parentheses ( ) for those variable that were present at the time of the study visit.

Results

The racial/ethnic composition of the SSc group was as follows: Caucasian, 47%; African American, 21%; Hispanic, 28% and others, 4%. The average age for SSc patients was 50.1±12.3 years. The percentage of females was 86%. The percentage with diffuse cutaneous involvement was 59%. The percentage who used tobacco (smoked cigarettes) was 17%. The average total skin score was 15.1±12.4.

Results of univariate analyses for plasma enkephalin levels, autoantibodies and clinical variables are shown in Table I. The plasma met-enkephalin levels were significantly lower in SSc patients who were seropositive for TOPO-I antibodies, compared to those who were seronegative (6.1±8.3 *vs*. 14.9±22.8 ug/ml, respectively, p=0.02). No significant correlations with plasma met- and leu-enkephalin levels were seen for samples seropositive for the following autoantibodies: antinuclear, anti-centromere, anti ribonucleotidase, Th/TO, polymerase I, polymerase II, polymerase III or anti-fibrillarin.

Mean plasma leu-enkephalin levels were significantly higher in the presence of digital pulp loss, compared to the patients without pulp loss, as shown (97.0±134 *vs*. 65.1±100 ug/ml, respectively, p=0.04). Mean plasma leu-enkephalin levels were significantly lower in the presence of myositis, compared to the patients without myositis, as shown (14.3±11.1 *vs*. 77.7±113 ug/ml, respectively, p=0.047). Only five patients had myositis at the time of the visit.

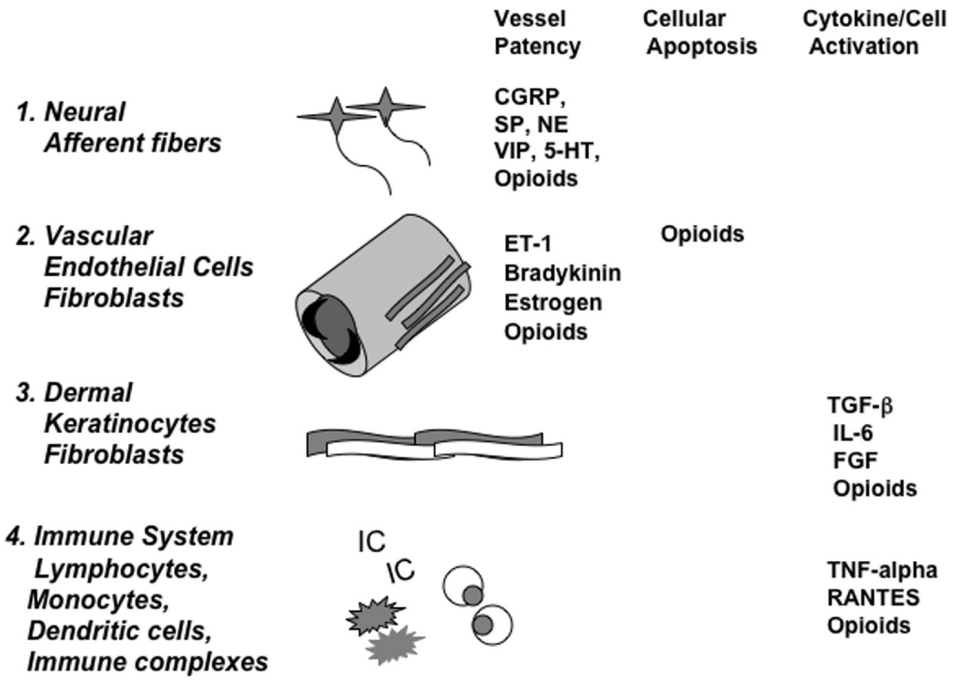

Fig. 1. Proposed cellular targets for endogenous enkephalins to contribute to vasogenic and fibrogenic pathologies in SSc. This figure illustrates enkephalin interactions with 1. neurogenic, 2. vascular, 3. dermal and 4. immunologic systems to promote SSc pathology via modulation of neurogenic, vasoactive, apoptotic and immunologic systems. CGRP: calcitonin gene-related peptide; SP: substance P; NE: norepinephrine; VIP: vasoactive intestinal peptide; ET-1: endothelin-1; BK: bradykinin; TGF-β: transforming growth factor-beta; IL-6: interleukin 6; FGF: fibroblast growth factor; IC: immune complex.

Mean plasma met-enkephalin levels were modestly decreased in patients who reported Raynaud's phenomenon (RP), compared to those who did not (12.8±21.4 *vs*. 16.6±5.9 ug/ml, respectively, p=0.07) The SSc patients with RP had increased plasma leu-enkephalin levels compared to the small number who did not, although the difference was not significant (78.2±114.8 *vs*. 25.9±29.2 mg/ml, p=0.1). SSc patients with RP (n=109) compared to those with no RP (n=7) at the time of study visit had higher frequencies of sclerodactyly (96% *vs*. 71%, p=0.042) and digital pits/ulcers (66% *vs*. 29%, p=0.097

Mean plasma met-enkephalin levels were also modestly decreased in the presence compared to absence of tendon friction rubs (3.5±3.2 *vs*. 14.0±21.7, p=0.06). Mean plasma leu-enkephalin levels were decreased in the presence compared to absence of telangiectasias (44.6±58.9 *vs*. 85.0±123.2, p=0.09). Minimal differences in mean plasma met-enkephalin levels were noted in dSSc patients compared to lSSc patients (12.5±16.4 *vs*. 13.8±26.0, p=1.0). Mean plasma leu-enkephalin levels were not significantly higher in dSSc patients compared to lSSc patients (84.4±128.1 *vs*. 64.6±90.6, p=0.72). No associations were noted between plasma enkephalin levels and other digital manifestations (total skin score, digital ulcers or digital gangrene), systemic manifestations, scores from the Scleroderma-HAQ, SF-36 surveys, or medications. The modified MSS (composite scores or scores from nine individual organ systems) did not associate with the plasma endogenous enkephalin levels in early SSc.

Significant effects noted by univariate analysis by t-test were not sustained after controlling for age, gender and race by multiple linear regression. Power analysis determined our sample size was not large enough to achieve 80% power at a 5% significance level for the association of TOPO-I, digital pulp loss or myositis with plasma enkephalin levels, (needed ≥190 patients, 14).

Discussion

These data are consistent with our hypotheses that decreased plasma met-enkephalin levels are vasogenic mediators in SSc, and will associate with clinical manifestations of SSc-related vascular and fibrogenic injuries. Plasma met-enkephalin levels were modestly to significantly lower in patients seropositive for TOPO-I and in patients with loss of digital pulp, RP, tendon friction rubs, myositis, telangiectasias and diffuse cutaneous involvement. Plasma leu-enkephalin levels were higher in patients seropositive for TOPO-1 and with loss of digital pulp, RP and tendon friction rubs. We predict that met-enkephalin levels will be inversely associated with vasogenic and fibrogenic activity in early SSc. It will be important to study plasma enkephalin fluctuations in larger group numbers over time in the context of SSc disease activity and progression.

Recent studies highlight an emerging appreciation for endogenous opioid and opioid receptor interactions in vasodilatory, anti-apoptotic and anti-inflammatory functions (6-8). Elevated endothelin-1 levels reported in SSc may mediate vasoconstriction in peripheral vessels, in part, by modulation of met-enkephalin. In a percussive brain injury model, endothelin receptor activation decreased met-enkephalin mediated cerebral blood flow. Application of an endothelin-1 antagonist restored enkephalin- induced vasodilation (8).

TOPO-1 autoantibody seropositivity is associated with skin and vascular changes (13). TOPO-1 autoantibodies have been reported to increase endothelial apoptosis, pro-fibrogenic activation and digital contractures (5, 15, 16). TOPO-1 immune complexes may

increase endothelin-1 and subsequently decrease met-enkephalin levels, to trigger or enhance cellular apoptosis in endothelial injury. SSc specific antibodies (*i.e.* anti-centromere antibodies or TOPO-1) may also contribute to peripheral tissue injury by autonomic dysfunction (17).

Enkephalin levels may also directly of indirectly impact fibrogenic processes. The association of higher plasma leu-enkephalin levels with loss of digital pulp in SSc patients supports their potential relevance in subsequent tissue remodeling from increased fibrogenic activity. Reported physiologic interactions of cytokines such as transforming growth factor-beta expression and opioid agonists possibly contribute to wound healing (18). Indirectly, lower met-enkephalin levels have been demonstrated to promote apoptosis via the cellular caspase-3 pathway in cultured cells (7). Serum autoantibodies from SSc patients are reported to increase apoptosis of cultured dermal endothelial cells by a similar mechanism (15). Fig. 1 illustrates selected proposed cellular targets of decreased endogenous enkephalin expression in vasogenic and fibrogenic injury.

Below are selected studies that support a proposed physiologic relevance of plasma endogenous opioid levels in SSc and their potential as therapeutic targets.

1. Circulating endogenous enkephalin levels may have modest physiologic influences under normal conditions, but become prominent regulators of vascular tone during episodes of injury or increased stress, such as persistent tissue hypoxia (8).

2. Mu opioid receptor agonists demonstrated vasoconstrictive properties at low and vasodilatory properties at high concentrations in cardiovascular models (19).

3. Supplementation with other neurotransmitter agonists has induced vasodilation in secondary Raynaud's and animal models, supporting a potential physiological impact of plasma mediators in peripheral tissue processes (3, 20).

4. Two-fold increased plasma β-endorphin levels in psoriatic patients (compared to normal controls) were attributed to reduced capsaicin-induced neurogenic inflammatory responses in psoriatic skin (21), supporting a physiologic (and potentially pathologic) consequence of plasma opioid levels.

5. A recent study has demonstrated that treatment with α-melanocyte stimulating hormone (a gene product of the POMC gene), suppressed skin fibrosis in a bleomycin mouse model of SSc (22). This supports a role of opioid related gene products in fibrotic processes.

6. Digital transcutaneous electrical nerve stimulation (TENS), which increases opioid peptide levels (reviewed in 23), promotes peripheral vasodilation, with improved blood flow to the digits (24). Electrical neural stimulation can promote selective release of neurotransmitters, including opioid peptides (25).

Plasma enkephalins and tissue opioid receptors may provide novel or additive therapeutic targets for restoring or increasing tissue oxygenation in the inflammatory and vasculopathic processes of anoxic injury. Studies with larger patient enrollment are needed to determine if plasma or tissue enkephalin levels are physiologically relevant and which mechanisms of action are most pertinent to scleroderma pathogenesis. Future studies with capillaroscopy, which is noninvasive, and enkephalin levels would be desirable as significant associations of enkephalin levels with parameters of vascular integrity would be predicted (26, 27). Ultimately, if plasma enkephalins are depleted by repeated episodes of vasospasm, SSc patient subsets with increased risk of hypoxic injury may need additional therapies to minimise vascular or fibrotic injury (28).

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References

1. DENTON CP, BLACK CM, ABRAHAM DJ: Mechanisms and consequences of fibrosis in systemic sclerosis. *Nat Clin Pract Rheumatol* 2006; 2: 134-44.
2. OSTOJI P, DAMJANOV N, PAVLOV-DOLIJANOVIC S, RADUNOVI G: Peripheral vasculopathy in patients with systemic sclerosis: difference in limited and diffuse subset of disease. *Clin Hemorheol Microcirc* 2004; 31: 281-5.
3. HERRICK AL: Pathogenesis of Raynaud's phenomenon. *Rheumatology* 2005; 44: 587-96.
4. HESSELSTRAND R, SCHEJA A, SHEN GQ, WIIK A, AKESSON A: The association of antinuclear antibodies with organ involvement and survival in systemic sclerosis. *Rheumatology* 2003; 42: 534-40.
5. RADI M, MARTINOVI KD, LJUTI D: The level of anti-topoisomerase I antibodies highly correlates with metacarpophalangeal and proximal interphalangeal joints flexion contractures in patients with systemic sclerosis. *Clin Exp Rheumatol* 2006; 24: 407-12.
6. MACHELSKA H, STEIN C: Immune mechanisms of pain and analgesia. Georgetown, TX: Landes Bioscience/Eurekah.com; 2003.
7. XU J, XIN S, LI H *et al*.: Involvement of caspase-3 pathway in anti-apoptotic action of methionine enkephalin on CEM × 174 cells in prolonged infection with simian immunodeficiency virus *in vitro*. *Cell Biol Intl* 2006; 30: 114-21.
8. KASEMSRI T, ARMSTEAD WM: Endothelin production links superoxide generation to altered opioid-induced pial artery vasodilation after brain injury in pigs. *Stroke* 1997; 28: 190-7.
9. REVEILLE JD, FISCHBACH M, MCNEARNEY T *et al*., GENISOS STUDY GROUP: Systemic sclerosis in 3 US ethnic groups: a comparison of clinical, sociodemographic, serologic, and immunogenetic determinants. *Semin Arthritis Rheum* 2001; 30: 332-46.
10. MASI AT, RODNAN GP, MEDSGER TA *et al*.: Preliminary criteria for the classification of systemic sclerosis (scleroderma) *Arthritis Rheum* 1980; 23: 581-90.
11. LEROY EC, BLACK C, FLEISCHMAJER R *et al*.: Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. *J Rheumatol* 1988; 15: 202-5.
12. Climate and topographical records from the National Oceanic Atmospheric Administration (NOAA), www.nws.noaa.gov/
13. LISI TL, SLUKA KA: A new electrochemical HPLC method for analysis of enkephalins and endomorphins. *J Neurosci Methods* 2006; 150: 74-9.
14. COHEN J: Statistical Power Analysis for the Behavioral Sciences. 2nd edition. Hillsdale, NJ: Lawrence Erlbaum Associates; 1988.
15. HANKE K, DÄHNRICH C, BRÜCKNER CS *et al*.: Diagnostic value of anti-topoisomerase I antibodies in a large monocentric cohort. *Arthritis Res Ther* 2009; 11: R28-R37.
16. AHMED SS, TAN FK, ARNETT, FC, JIN, L, GENG, Y-J: Induction of apoptosis and fibrillin 1 expression in human dermal endothelial cells by scleroderma sera containing anti-endothelial cell antibodies. *Arthritis Rheum* 2006; 2250-62.
17. HENAULT J, ROBITAILLE G, SENECAL J-L, RAYMOND Y: DNA topoisomerase I binding to fibroblast induces monocyte adhesion and activation in the presence of anti-topoisomerae-I autoantibodies from systemic sclerosis patients. *Arthritis Rheum* 2006; 54: 963-73.
18. BERTINOTTI L, BRACCI S, NACCI F *et al*.: The autonomic nervous system in systemic sclerosis. A review. *Clin Rheumatol* 2004; 23: 1-5.

19. CHATZAKI E, MARGIORIS AN, MAKRIGIANNAKIS A, CASTANAS E, GEORGOULIAS V, GRAVANIS A: Kappa opioids and TGF beta1 interact in human endometrial cells. *Mol Hum Reprod* 2000; 6: 602-9.
20. RAO SP, CONLEY A, DUNBAR JC: Cardiovascular responses to central administration of mu and kappa opioid receptor agonists and antagonist in normal rats. *Peptides* 2003; 24: 745-54.
21. GLINSKI W, BRODECKA H, GLINSKA-FERENZ M, KOWALSKI D: Increased concentration of beta-endorphin in sera of patients with psoriasis and other inflammatory dermatoses. *Br J Dermatol* 1994; 131: 260-4.
22. KOKOT A, ANCA SINDRILARU A, SCHILLER M *et al*.: Alpha-melanocyte-stimulating hormone suppresses bleomycin-induced collagen synthesis and reduces tissue fibrosis in a mouse model of scleroderma: Melanocortin peptides as a novel treatment strategy for scleroderma? *Arthritis Rheum* 2009; 60: 592-603.
23. SLUKA KA, WALSH D: Transcutaneous electrical nerve stimulation: Basic science mechanisms and clinical effectiveness. *J Pain* 2003; 4: 109-21.
24. CRAMP AF, GILSENAN C, LOWE AS, WALSH DM: The effect of high- and low-frequency transcutaneous electrical nerve stimulation upon cutaneous blood flow and skin temperature in healthy subjects. *Clin Physiol* 2000; 20: 150-7.
25. HAN JS: Acupuncture: neuropeptide release produced by electrical stimulation of different frequencies. *Trends Neurosci* 2003; 26: 17-22.
26. RICCIERI V, GERMANO V, ALESSANDRI C *et al*.: More severe nailfold capillaroscopy findings and anti-endothelial cell antibodies. Are they useful tools for prognostic use in systemic sclerosis? *Clin Exp Rheumatol* 2008; 26: 992-7.
27. CUTOLO M, PIZZORNI C, SECCHI ME, SULLI A: Capillaroscopy. Best Pract *Res Clin Rheumatol* 2008; 22: 1093-108.
28. WALKER UA, TYNDALL A, CZIRJAK L *et al*.: Geographic variation of disease manifestations in systemic sclerosis – a report from the EULAR Scleroderma Trials And Research (EUSTAR) group data base. 2008; *Ann Rheum Dis* 2009; 68: 856-62.