Differential association of the N-propeptide of collagen IIA (PIIANP) and collagen II C-telopeptide (CTX-II) with synovitis and erosions in early and longstanding rheumatoid arthritis

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Abstract
Objectives
To determine the N-terminal propeptide of collagen IIA (PIIANP) in early and established rheumatoid arthritis (RA) and to study the association with collagen II degradation assessed by its C-telopeptide (CTX-II), x-ray status and disease activity measures.

Methods
Two cohorts of RA patients were included: A) a one-year prospective cohort including 45 patients with early, untreated RA and B) a cross-sectional study comprising 50 RA patients with advanced disease. Blood donors and healthy volunteers served as controls. PIIANP in serum and urine CTX-II were measured by ELISA.

Results
PIIANP did not differ from control levels at any time in patients with early RA (p=0.16 and p=0.89), but at one-year follow-up, PIIANP was decreased compared with baseline (p=0.046). In patients with longstanding RA, PIIANP was lower than in controls (p=0.002) and RA patients with a 12-month disease (p=0.01). PIIANP was unrelated to joint counts and CRP in both cohorts, but baseline PIIANP was lower among x-ray progressors than in non-progressors (p=0.04). CTX-II was persistently increased in both cohorts (p<0.001 and p<0.001). CTX-II was positively associated with joint counts and CRP but not with x-ray progression (p=0.84). There was no correlation between PIIANP and CTX-II.

Conclusion
Declining PIIANP with increasing RA duration and persistently increased CTX-II indicate that cartilage anabolic and degradative pathways are unbalanced from clinical RA onset. Furthermore, that collagen II depletion in RA is both mediated by anti-anabolic effects unassociated with synovitis (decreased PIIANP) and by excess collagen II degradation linked to synovitis (increased CTX-II).

Key words
PIIANP, CTX-II, cartilage, rheumatoid arthritis.
PIIAP and CTX-II in early and longstanding rheumatoid arthritis / A.F. Christensen et al.

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Background
The majority of patients with rheumatoid arthritis (RA) will develop irreversible joint deformities unless tight synovitis control can be achieved (1). This is particularly important in the first few months after clinical diagnosis which seems to represent an important window of therapeutic opportunity regarding the effect of traditional anti-rheumatic agents (2, 3). Increased knowledge about the pathophysiologic processes in the rheumatoid joint, the advent of potent targeted new therapies and improved imaging techniques call for the development of candidate biomarkers for specific aspects of the disease process for use in the clinic as supplement to systemic measures of inflammation, C-reactive protein (CRP) in particular.

Cartilage degradation is a key feature of rheumatoid joint inflammation. Accordingly, an array of assays has been developed for the assessment of cartilage synthesis and breakdown (4). However, so far, no single biomarker has proven sufficiently accurate for routine clinical purposes. Furthermore, current clinical evaluations of existing markers do not allow unequivocal determination of cut-off levels and appropriate intervention responses, which will be required for eventual introduction of the markers in routine clinical practice.

Collagen type II is the major structural protein in cartilage, and soluble fragments of this protein are supposed to reflect the metabolic processes in joint cartilage (4). The C-telopeptide fragment of type II collagen (CTX-II) is currently the prevailing marker for collagen II degradation in osteoarthritis (OA) and RA (5). The propeptides of collagen type II are released from the protein during its synthesis and incorporation into the cartilage matrix, and can be used as measures of matrix synthesis. The role of the C-terminal propeptide of collagen II (CPII), for the assessment of cartilage collagen synthesis is less well defined at the present time (4), but a recent study on OA patients supports the notion that CPII reflects collagen II synthesis (6).

Type II procollagen exists in two forms as a result of alternative splicing (7). While synthesis of collagen type IIB prevails in mature cartilage, collagen IIA is predominantly generated during development in immature cartilage and in non-chondrogenic premature tissues like nerves and internal organs (8-11). This collagen type II variant comprises an additional cystein-rich domain of 69 amino acids in the N-terminal propeptide. The vitreous body is the only adult human healthy tissue known to express substantial amounts of type IIA collagen (12). However, type IIA collagen is re-expressed in damaged cartilage, e.g. OA, in chondrosarcomas and other malignant tumours (13-16). As expression of type IIA collagen seems to be a hallmark of pathological changes in cartilage, measurement of PIIAP in biological fluids has been proposed as an anabolic marker in joint diseases (17-19).

It was recently reported that PIIAP in serum is decreased in patients with OA and established RA. The authors speculate that these findings reflect inhibitory effects on cartilage metabolism by pro-inflammatory cytokines (18).

Based on the assumption, that cartilage anabolic and catabolic pathways are unbalanced in favour of increased breakdown at different stages of the disease, the purpose of the present investigation was to study two markers of collagen type II metabolism, PIIAP and CTX-II in a prospective study on newly diagnosed, DMARD (disease modifying anti-rheumatic drugs) naïve RA patients and in the same setting to measure PIIAP in a well-defined cohort of RA patients with advanced disease.

Methods
Patients
The study included two subsets of RA patients:

- a) Newly diagnosed RA: a one year prospective study including 45 DMARD naïve patients with newly diagnosed RA, disease duration less than 12 months. The patients were followed for 1 year on methotrexate starting at 7.5 mg increasing to maximum 20 mg per week. Patients with high disease activity and unable to await the onset of methotrexate action at inclusion were excluded. Methotrexate was offered prednisolone 7.5 mg orally per day (4 and 6 patients at baseline and year 1, respectively).

- b) Newly diagnosed RA: a one year prospective study including 45 DMARD naïve patients with newly diagnosed RA, disease duration less than 12 months. The patients were followed for 1 year on methotrexate starting at 7.5 mg increasing to maximum 20 mg per week. Patients with high disease activity and unable to await the onset of methotrexate action at inclusion were offered prednisolone 7.5 mg orally per day (4 and 6 patients at baseline and year 1, respectively). The patients all met the 1987 American College of
Rheumatology (ACR) criteria for classification of RA (20). Additional inclusion criteria were disease duration less than 12 months and age 18-71 years. Exclusion criteria were a previous history of arthritis and treatment with DMARDs, past or present malignancy, kidney or liver disease, oral or intraarticular glucocorticoid therapy within 4 weeks prior to inclusion.

b) Advanced RA: a cross-sectional study comprising 50 patients with established RA. Inclusion criteria were fulfillment of the revised ACR 1987 criteria for RA, disease duration of 5-15 years, erosions by x-ray in at least 1 joint/joint area, age 18-75 years. Exclusion criteria were glucocorticoid therapy within 4 weeks, DMARD therapy change within 3 months, surgery within 8 weeks, past or present malignancy, liver and/or kidney disease, serious heart (NYHA class III-IV) or lung disease, advanced atherosclerosis, anticoagulant therapy, concomitant inflammatory rheumatic disease, arterial hypertension (>140/90 mmHg despite antihypertensive treatment), pregnancy and lactation. All patients were on a DMARD (methotrexate (19) as mono-therapy and 12 as part of combination therapy), leflunomide (4), aurothiomalate (5), sulfasalazine (6), sulfasalazine+azathioprine (1), azathioprine (1), penicillamine (1) and hydroxychloroquine (1)). None were treated with glucocorticoids. The patients were recruited on a voluntary basis and both in- and outpatients participated.

Controls
Two control groups were used: 1) One hundred and twenty blood donors, comprising 60 (50%) women, served as controls for PIIANP measurements. The controls were divided into decades with 24 controls in each decade (2-6) at inclusion. Only sex and age-group were available in this cohort. 2) Six hundred and thirty six healthy in- and outpatients participated. The controls were divided into 2 groups: 50 patients with established RA. Inclusion criteria were fulfillment of the revised ACR 1987 criteria for RA, disease duration of 5-15 years, erosions by x-ray in at least 1 joint/joint area, age 18-75 years. Exclusion criteria were glucocorticoid therapy within 4 weeks, DMARD therapy change within 3 months, surgery within 8 weeks, past or present malignancy, liver and/or kidney disease, serious heart (NYHA class III-IV) or lung disease, advanced atherosclerosis, anticoagulant therapy, concomitant inflammatory rheumatic disease, arterial hypertension (>140/90 mmHg despite antihypertensive treatment), pregnancy and lactation. All patients were on a DMARD (methotrexate (19) as mono-therapy and 12 as part of combination therapy), leflunomide (4), aurothiomalate (5), sulfasalazine (6), sulfasalazine+azathioprine (1), azathioprine (1), penicillamine (1) and hydroxychloroquine (1)). None were treated with glucocorticoids. The patients were recruited on a voluntary basis and both in- and outpatients participated.

Clinical evaluation
Systematic clinical evaluation was made by one experienced rheumatologist at inclusion and after 52 weeks in cohort A and at inclusion in cohort B. Swollen and tender joint counts and functional impairment score (health assessment questionnaire, HAQ (21)) were computed.

X-ray
Radiographs of hands (posteroanterior and Nørgaard projections (22)) and wrists (posteroanterior and lateral projections) were obtained in both patient cohorts. Radiographs were obtained at baseline and after 12 months in patients with early RA. Patients with advanced disease were x-rayed at inclusion. The radiographs were evaluated by one experienced senior musculoskeletal radiologist and 14 joints including 2 wrists, 10 metacarpophalangeal joints and 2 interphalangeal joints (thumbs) were scored according to the Larsen method (23, 24). In early RA, patients were divided into radiographic progressors and non-progressors, the former being defined as patients with higher erosion score at 1 year compared to baseline.

Laboratory analyses
Non-fasting blood was collected between 8.00 am and 2.00 pm, at least one hour after bed rest. Samples were centrifuged at 4000 g for 10 min and stored at -80°C. Spot urine samples were collected as the second, non-fasting urine void in the morning and kept frozen at -20°C until analyzed.

PIIANP was measured by a competitive ELISA (LINCO Research, USA) using a polyclonal antibody raised against recombinant GST-human type II procollagen 2 fusion protein specific for the N-propeptide of type IIA collagen (9). Homologous serum proteins did not cross-react with this polyclonal antibody (25). The inter-assay coefficients in our laboratory were 17.1% and 10.8% for low and high concentrations, respectively. If more than two paired samples from the same patient were analyzed simultaneously, the intra-assay variation was <5%. All analyses were done in duplicate using kits with the same lot number and serial samples from the same patient were analyzed simultaneously.

Urine CTX-II was assayed by ELISA (Nordic Bioscience, Denmark) as previously described (5, 26). Intra- and inter-assay variation amounted to 7.6% and 8.3%, respectively.

CRP and erythrocyte sedimentation rate (ESR) were measured using standard methods and IgM-rheumatoid factor was determined by ELISA (27).

Ethics
The study was conducted in accordance with the Declaration of Helsinki, and local ethics committee approval was obtained before the study start (J. No 19980024 and M-2359-02).

Statistical analyses
Mann-Whitney U-test and Fisher’s exact test were used to compare groups for non-dichotomous and dichotomous responses, respectively. If more than two groups, the Kruskal-Wallis test was used. Adjustment for sex and age was done using multiple regression models. When including both RA patients and controls in the regression models adjustment for disease status was done. The analysis of prospective data was carried out by linear regression models using STATA’s cluster option to support the assumption of independence within groups, i.e. treating only observations with differing person id as truly independent. PIIANP and CTX-II were logarithmically transformed to approximate normal distribution when used as a dependent variable in linear regression models. Associations between PIIANP or CTX-II, disease activity, radiology and marker molecules were done by Spearman Rank correlation and Mann-Whitney U-test. Logistic regression was used to assess the risk of radiographic progression with adjustment for sex, age, erosive status, CRP and IgM-RF at baseline. In addition, odds ratios (OR) were calculated for the change in risk per standard deviation (SD) of the mean value in controls. The results are presented as median (95 % confidence interval) if not otherwise stated. ps0.05 were regarded as significant.

All analyses were conducted in STATA/ version 9 (StataCorp, Texas, USA)

Results
Controls
PIIANP normal range in serum was 284-2356, median 895 ng/ml. Male
blood donors had significantly higher levels of PIIANP than females (941 [883; 1021] vs. 870 [797; 930] ng/ml, p=0.03). PIIANP levels did not differ between age-groups (p=0.8), but women <50 years (n=36) had significantly lower PIIANP than women >50 years (n=12) (859 [737; 933] vs. 962 [887; 1156] ng/ml, p=0.03).

The normal range for urinary CTX-II was 0.02 - 1.59, median 0.14 μg/mmol creatinine. While no gender differences were observed (p=0.49), CTX-II varied significantly between age-groups (p=0.001). This variation was least prominent in subjects in their forties and did not change after stratification for gender (p=0.01 for men and women, respectively). Stratification of women aged 49 to 53 according to menopausal status showed that postmenopausal women (n=221) had significantly higher levels of CTX-II than premenopausal women (n=162) (0.17 [0.16;0.19] vs. 0.12 [0.11; 0.13] μg/mmol creatinine, p<0.001).

**RA clinical variables**

No difference between the two cohorts was observed regarding sex, age and rheumatoid factor status. Patients with early disease had higher CRP, tender and swollen joint counts and HAQ score at baseline than patients with longstanding disease. Baseline characteristics of both cohorts are outlined in Table I.

**RA biochemistry**

In early RA, three patients were excluded from the statistical analyses to reduce skewness of both non-logarithmically and logarithmically transformed data (2 patients had baseline PIIANP levels of 5946 ng/ml and 3613 ng/ml, respectively, and one patient had CTX-II level of 6.65 μg/mmol creatinine). PIIANP was not available in one patient with longstanding disease. No difference in disease activity or erosion status between these patients and the remaining patients was observed (data not shown).

At disease onset and after a one-year follow-up, PIIANP did not differ significantly from controls: 967 (880; 1074) vs. 895 (870; 950) and 919 (841; 1007) vs. 895 (870; 950) ng/ml, p-adjusted=0.16 and p-adjusted=0.89, respectively. However, by one year PIIANP was significantly decreased as compared with baseline (Fig. 1). This trend remained after adjustment for gender and age (βPIIANP=-0.09 [-0.18; 0.001], p-adjusted=0.053). In patients with advanced disease PIIANP was even more decreased both when compared to controls (790 [670; 868] vs. 895 [870; 950] ng/ml, p-adjusted=0.002) and RA patients with 12 months disease duration (p-adjusted = 0.01) (Fig. 1). In contrast to healthy subjects, PIIANP did not differ between female and male RA patients at any stage of the disease (early RA patients at baseline: 960 [647; 1361] ng/ml in men vs. 967 [877;1118] ng/ml in women, p=0.53 and Cohort B: 718 [589; 979] ng/ml in men vs. 791 [671;878] ng/ml in women, p=0.74 or within age-groups (p=0.71 and p=0.58, respectively). Stratification according to menopausal status (women aged <50 years vs. women aged >50 years) revealed no difference in PIIANP. This observation applied to both RA cohorts (p=0.43 and p=0.28, respectively).

CTX-II was elevated more than twofold at baseline and remained high after one year in patients with early RA (baseline: 0.30 (μg/mmol creatinine) (0.21; 0.45) and 1 year: 0.33 (μg/mmol creatinine) (0.18-0.40) vs. 0.14 (μg/mmol creatinine) (0.13; 0.14), p-adjusted p<0.001 and p<0.001, respectively) compared with controls (Fig. 2). The CTX-II level after one year did not differ significantly from baseline CTX-II (βbaseline=-0.02 [-0.3; 0.34], p=0.90), even after adjustment for sex and age (data not shown).

Similarly, CTX-II was higher in RA patients with longstanding disease compared to control subjects after adjustment for sex and age (0.25(μg/mmol creatinine) [0.21; 0.31] vs. 0.14 (μg/mmol creatinine) [0.13; 0.14], p<0.001) (Fig. 2). CTX-II did not differ between RA patients at disease onset or one-year

### Table I. Demographic, clinical and laboratory characteristics of patients with early and longstanding RA.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Early RA</th>
<th>Longstanding RA</th>
<th>p-value**</th>
</tr>
</thead>
<tbody>
<tr>
<td>n.</td>
<td>45</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Disease duration*</td>
<td>&lt; 12 months</td>
<td>9 years (5.5)</td>
<td></td>
</tr>
<tr>
<td>Age at inclusion in years*</td>
<td>55 (17)</td>
<td>58 (14)</td>
<td>0.09</td>
</tr>
<tr>
<td>Sex (women/men)</td>
<td>31/14</td>
<td>32/18</td>
<td>0.67</td>
</tr>
<tr>
<td>Patients with erosions (%)</td>
<td>6 (13) (at disease onset)</td>
<td>50 (100)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IgM-rheumatoid factor positive (%)</td>
<td>35 (78)</td>
<td>44 (88)</td>
<td>0.27</td>
</tr>
<tr>
<td>HAQ score (0-3)*</td>
<td>0.55 (0.75)</td>
<td>0.38 (0.63)</td>
<td>0.03</td>
</tr>
<tr>
<td>CRP (mg/l)*</td>
<td>18 (36)</td>
<td>13 (12)</td>
<td>0.01</td>
</tr>
<tr>
<td>Swollen joints*</td>
<td>9 (8)</td>
<td>3 (2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tender joints*</td>
<td>6 (9)</td>
<td>3 (5)</td>
<td>0.01</td>
</tr>
<tr>
<td>Serum PIIANP (ng/ml at disease onset)</td>
<td>967 [880;1074]</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Serum PIIANP (ng/ml) after 1 year.</td>
<td>919 [841;1007]</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Serum CTX-II (μg/mmol creatinine) at disease onset</td>
<td>0.30 [0.21;0.45]</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CTX-II (μg/mmol creatinine) after 1 year</td>
<td>0.33 [0.18;0.40]</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Median (interquartile range) otherwise median [95 % confidence interval].
**Calculated using Fishers exact test or Mann Whitney U-test.

HAQ: health assessment questionnaire; CRP: C-reactive protein; PIIANP: N-terminale propeptide of collagen type IIA; CTX-II: C-telopeptide fragment of collagen II.
follow-up compared to longstanding disease ($p_{\text{adjusted}}=0.52$ and $p_{\text{adjusted}}=0.58$, respectively).

There were no gender differences with respect to CTX-II in RA patients at baseline, nor at one year follow up or in longstanding disease ($p=0.51$, $p=0.67$ and $p=0.43$, respectively). A similar pattern was observed in the control cohort. CTX-II levels did not appear to be significantly affected by menopausal status in either cohort, but the limited number of definite pre-and post menopausal women in the two RA populations should be considered.

No correlation was observed between PIIANP and CTX-II at baseline and after one year in early RA (rho=-0.15, $p=0.36$ and rho=-0.04, $p=0.82$, respectively) or longstanding RA (rho=-0.05, $p=0.72$). There was no correlation between Δ-PIIANP and Δ-CTX-II in early RA (rho=0.25, $p=0.14$).

**Correlations with clinical and x-ray findings**

No correlation was observed between baseline or one year PIIANP and tender and swollen joint count, CRP or x-ray findings (data not shown). In addition, PIIANP did not differ between rheumatoid factor positive and negative individuals at baseline ($p=0.55$). However, patients who progressed radiographically (n=8) had significantly lower PIIANP at baseline compared with non-progressors (736 ng/ml [587;1247] vs. 989 ng/ml [900;1181], $p=0.04$). Conversely, decreased baseline-PIIANP, defined as -1 SD (361.5 ng/ml) of the mean value in controls, could not predict radiographic progression after adjustment for potential confounders (OR: 1.78 [0.85;3.71], $p=0.12$).

Demographic characteristics of progressors and non-progressors are outlined in Table II from which it appears that progressors have slightly higher joint counts than non-progressors. The change in PIIANP from baseline to 1 year did not correlate with the change in any of the outcome variables in patients with early RA (Table III). There was no correlation between PIIANP and laboratory or clinical variables in patients with longstanding RA (Table III). The change in tender and swollen
Tender joints (rho=0.04, p=0.006). No association to erosions at baseline or after 1 year was observed (data not shown). CTX-II at baseline did not differ between erosive and non erosive patients (Mann-Whitney U-test, p=0.84). Similarly, after adjustment for potential confounders, elevated baseline CTX-II, defined as +1 SD (0.13 μg/mmol creatinine) from the mean in the controls, did not predict subsequent radiographic progression during the one-year observation period (OR: 0.72 [0.48; 1.09], p=0.12).

**Discussion**

This study is the first to measure circulating PIIANP prospectively in RA from clinical disease onset and 12 months on. While the levels of this putative marker of cartilage formation did not differ between newly diagnosed RA patients and healthy controls at inclusion, PIIANP was significantly decreased by 12 months as compared with baseline, and PIIANP was even lower in patients with longstanding disease. PIIANP was un-related to joint counts and CRP at any time in both cohorts. Baseline PIIANP was significantly lower in x-ray progressors as compared with non-progressors, although radiographic progression could not be predicted by PIIANP levels. By contrast, urinary CTX-II was equally increased in both RA subsets, and CTX-II was positively correlated with joint counts and CRP. There was no correlation between CTX-II and PIIANP at any time in either cohort.

Our data on PIIANP are in line with a recent report based on longstanding RA (18). These authors proposed that decreased PIIANP could result from increased expression of pro-inflammatory cytokines in the inflamed joint. Our observation of decreased PIIANP by 12 months despite excellent synovitis control as judged clinically indicates an anti-anabolic effect on cartilage by mechanisms which are not detected by traditional measures of RA disease activity. This accords with evidence, that erosions may develop in spite of clinical remission (28, 29), probably due to a local pro-inflammatory environment caused subclinical synovitis (30).

When interpreting these results the following issues should be considered. First, the control subjects had higher levels of PIIANP compared to RA patients with advanced disease. This finding could reflect that PIIANP is also released from extra-articular sites, suggesting that synthesis of collagen IIA also occurs in healthy persons. In the Genetics osteoarthritis and Progression (GARP) study, Meulenberg et al. found that PIIANP expression was associated with spine and/or hand OA when clustered with cartilage oligomeric matrix protein and age, suggesting that the spine may be a major contributor to PIIANP in serum (31).

Secondly, both PIIANP and CTX-II exhibit diurnal variation at approximately
20% (5, 32, 33). However, in the present study, PIIANP was decreased in RA patients, although PIIANP has been reported to increase during morning activities in OA (33). CTX-II has been shown to decrease slightly during the daytime (5; 32). Such an effect was not apparent among our RA patients.

On the contrary, like in previous studies, CTX-II was consistently increased from clinical disease onset in RA patients, which is in contrast to previous studies (5, 34, 35). However in the present investigation, radiographs of hands were only scored according to the number of erosions. Besides, relatively few patients showed radiographic progression in the early RA cohort. An effect by glucocorticoid treatment is unlikely because only 4 and 6 patients were on low-dose prednisolone daily at baseline and 1-year follow-up.

PIIANP and CTX-II have been studied simultaneously in two OA cohorts (17, 19) but not in RA. Garnero et al. reported that high CTX-II and low PIIANP at baseline could identify OA patients at high risk of rapid x-ray and arthroscopic progression (17). It has recently been reported, that PIIANP and CTX-II epitopes rarely co-localize in OA cartilage estimated by immunohistochemistry (36). If this observation also applies to rheumatoid arthritis, such a spatial segregation of cartilage metabolic epitopes lends further support to the hypothesis, that the generation and release of these different cartilage metabolic markers are regulated by different and independent processes (29, 37, 38).

While PIIANP was significantly higher among healthy males than in women, no gender differences were observed between RA patients with short and long disease duration. In addition, healthy women aged >50 years had significantly higher PIIANP than women below 50 years. This age difference was not found in RA patients with early or longstanding disease, thereby supporting that the anabolic capacity of RA cartilage is compromised.

The progressive decrease of PIIANP despite clinically significant DMARD effects may reflect that these agents are unable to revert the imbalance between cartilage formation and degradation supposed to underlie the development of erosions.

In conclusion, this study provides additional evidence, that collagen II anabolic and degradative pathways are unbalanced from the clinical onset of RA. In addition, these results indicate, that collagen II depletion in RA is both mediated by anti-anabolic effects un-associated with synovitis (decreased PIIANP) and by excess collagen II degradation linked to synovitis (increased CTX-II).

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References


