Preclinical biomarker associations with both incident rheumatoid arthritis and its subsequent mortality: sex effects in a 41-year, community-based, case-control cohort study

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Abstract

Objective

To identify sex effects and preclinical serum biomarker associations with both incident rheumatoid arthritis (RA) and its subsequent mortality, using a 41-year, community-based, case-control cohort.

Methods

After cohort entry in 1974, incident RA cases (n=54) had clinical onsets between 1977 and 1994. Cohort control (CN) subjects were individually matched on entry to cases (4 CN:1 RA, n=216). All subjects were followed for survival from 1995 through 2015. Ranks (1–5) of preclinical z-scores within each set of 1 RA and 4 matched CN were analysed for associations with incident RA and mortality. Survival was evaluated using Cox proportional hazards models.

Results

Preclinical serum IgG RF z-score ranks associated with incident RA in 90 males (18 RA, 72 CN). Cigarette smoking, androstenedione, pregnenolone, and sIL-2Rα ranks associated with incident RA in 180 females (36 RA, 144 CN). Total percentile mortality was greater (p=0.003) in RA (70.4) vs. CN (49.9) and equivalently increased in female RA (69.4) vs. CN (49.3) and in male RA (72.2) vs. CN (43.1) subjects. Percentile respiratory-related CODs were greater (p=0.009) only in the female RA cases (16.7) vs. CN (3.5). Ranks of preclinical hsCRP (p=0.028) and sIL-2Rα (p=0.030) independently associated with 140 total deaths, as did sTNF-R1 (p=0.003) and hsCRP (p=0.005) with 50 CVD deaths. Latter biomarker association were significant in females. Therapy responses in 1995 significantly associated with subsequent mortality.

Conclusion

Sex effects were important in preclinical biomarker associations with incident RA, total and CVD mortality as well as occurrence of respiratory deaths.

Key words

rheumatoid arthritis, biomarkers, mortality, incidence, sex effects

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Received on January 28, 2017; accepted in revised form on April 3, 2017.

Competing interests: none declared.

Introduction

Rheumatoid arthritis (RA) is a multifactorial disease of unknown aetiology influenced by interaction of genetic, demographic, and environmental factors (1-4). Aging and female sex predispose to RA (1) as does heavy cigarette smoking in females (1, 3). Mortality is generally reported to be greater in RA than control (CN) populations (5-8), but not found in three recent studies (9-11). Cardiovascular disease (CVD) mortality is also reported to be greater in RA *versus* CN subjects in some (7, 12), but not in recent (9-11) studies. Elevations of serum inflammatory biomarkers have been associated with CVD in inflammatory polyarthritis (13) and RA patients (13-16) and in the general population (17, 18). Patients with greater RA disease activity or severity were reported to have increased total mortality (19-21) and a greater risk of cardiovascular events (22).

In one long-term follow-up study from 1978 to1998 of established RA patients (14 years mean disease duration), standardised mortality ratios were similarly increased in males and females (20). However, a Finnish nation-wide inception cohort of RA patients followed for a median (range) duration of 4 (1-9) years revealed significantly greater cumulative incidence of CVD mortality in men than women (23). A PubMed search of the key words "rheumatoid arthritis" and "sex effects" did not identify any human clinical report. Also, this relation was not included in a recent survey of systematic reviews of chronic pain patients (24). To our knowledge, the current study is the first investigation of sex effects and preclinical serum biomarkers associated with both incident RA and its subsequent total and CVD mortality in the same long-term population cohort.

Materials and methods

In 1991, the RA Precursors Study (RAPS) was initiated at this institution, approved 02/05/2016 by the University of Illinois College of Medicine at Peoria IRB 1 (IRB no. 00000688) for Study Title: (85786-5) Rheumatoid Arthritis Precursors Study (RAPS) Database Project. The incident RA cases and matched non-RA CN subjects were participants within CLUE I. This cohort enrolled adult community-based subjects (n=21,061) in 1974 and is operated by the Johns Hopkins Bloomberg School of Public Health. The incident RA cases in the cohort were identified in the practice of the sole community rheumatologist (25-27). Four CN subjects were selected from the 1974 CLUE I cohort for matching on entry characteristics to each RA case (4 CN: 1 RA) on age, sex, race (all Caucasian) and closest date of enrolment. The primary purpose of operation CLUE I is to study cancer biomarkers and risk factors (28-30). Female RA and CN subjects who had a known cancer diagnosis in biennial follow-up to 1992 were not included in the RAPS study.

After the 1974 CLUE I enrolment, the incident RA cases had clinical onsets between 1977 and 1994, 3 to 20 (mean 12) years (25-27). All RA cases were diagnosed and confirmed by the sole community rheumatologist and fulfilled American College of Rheumatology (ACR) 1987 revised classification criteria (31) and satisfied European League Against Rheumatism (EULAR) recommendations for pre-disease criteria at 1974 cohort entry (32). Laboratory assays were performed on coded sera in matched sets (n=54) consisting of one RA and four CN subjects, as previously described (25-27). Baseline (1974) stored (-70ºC) sera were blindly assayed for immunological biomarkers (ELISA kits, R&D, Minneapolis, MN), including: two acute phase proteins (APPs), ASAA and hsCRP; three inflammatory cytokines, IL-1β, IL-6, TNF-α; two cytokine receptors, sIL-2Rα and sTNF-R1, and a receptor antagonist, IL-1ra (26, 27). Adrenocorticosteroids and sex hormones were also blindly assayed (RIA), as previously described (25).

Statistical analysis

Baseline, preclinical serum biomarker values were transformed to natural logs and standardised by z-scores, within 3 separate subject subgroups: (1) earlier recruited women (1992); (2) later recruited women (1994), and (3) males (1995) as well as combined within the

total subjects (27). The minority of missing steroid values in females were imputed within their respective subgroups. Individual biomarker z-score values were analysed as ranks (1–5) within separate sets of 1 RA and 4 CN, as recently described (33). The set rank analysis method can reduce variation related to subject selection factors (*e.g.* pre- *vs*. post-menopausal) and batch effects on laboratory assays (34). The ranked z-scores of preclinical biomarkers were entered individually and in combination into multivariate models, including demographic covariates, as recently described (33). Logistic regression was used to identify preclinical variables which associated with incident RA *versus* CN status as the dependent outcome. In 1995, the incident RA cases were categorised by the sole community rheumatologist into 3 patterns of course-wide therapy response (Rxresp), as recently described (33). The therapy responses (good=1, moderate=2, and limited=3) were analysed as an independent variable with other covariates to determine association with mortality outcomes in Cox proportionate hazards models (33). The therapy response of each RA case was also assigned individually to the 4 CN subjects within each respective matched set, to evaluate any selective influence of therapy responses on the control subjects. No significant effects were found in any control subgroup analysis. Elapsed time from 1995 to mortality was assessed as the dependent variable in Cox proportional hazards regression. The independent variables in the models were demographic covariates, including deciles of age, sex, years of education completed, and daily cigarette smoking (0=non-smoker, 6=greater than 50+); the CN *versus* RA status; the 3 Rxresp categories, and set ranks of selected preclinical serum biomarkers. All biomarkers identified as significant ($p<0.050$) in analyses using rank values were confirmed using continuous log-transformed z-scores. A 3-category gradient of two combined dichotomous (neg *vs*. pos) z-scores of serum hsCRP and sTNF-R1 z-scores was created (0=neither positive, 1=either positive, 2=both positive) to further evaluate associations of serum inflammatory biomarkers with CVD mortality. Statistical analysis was performed using SPSS statistical software® (SPSS Incorporated, Chicago, Illinois). In this exploratory study, a significance level of $p\leq0.050$ was accepted without adjustment for multiple comparisons (35).

Table I. Baseline cohort entry (1974) biomarker and demographic variables which predicted incident rheumatoid arthritis (1977-94) cases from age- and sex- matched cohort control (cn) subjects.

	Females (36 RA *vs* 144 CN)	
Predictor variables in females	Exp β (±SE)	*p*
Ranks of Androstenedione	0.69 (0.51-0.94)	0.018
Ranks of Pregnenolone	1.41 (1.05-1.90)	0.025
Ranks of sIL-2-Rα	1.44 (1.06-1.95)	0.019
Age (deciles)	1.04 (0.72-1.51)	0.831
Cigarettes smoked daily (0-6 scale)	1.78 (1.24-2.57)	0.002
Education completed (yrs)	0.90 (0.75-1.08)	0.261
	Males (18 RA *vs* 72 CN)	
Predictor variables in males	Exp β (±SE)	*p*
Ranks of IgA Rheumatoid Factor*	1.27 (0.80-2.00)	0.316
Ranks of IgG Rheumatoid Factor†	1.70 (1.07-2.70)	0.025
Ranks of IgM Rheumatoid Factor‡	1.29 (0.81-2.04)	0.279
Age (deciles)	0.86 (0.43-1.73)	0.675
Cigarettes smoked daily (0-6 scale)	1.24 (0.91-1.69)	0.175
Education completed (yrs)	1.06 (0.87-1.30)	0.545
*IgA RF as sole biomarker in model	1.57 (1.05-2.36)	0.029
†IgG RF as sole biomarker in model	1.92 (1.23-3.01)	0.004
‡IgM RF as sole biomarker in model	1.61 (1.06-2.44)	0.024

Results

In multivariate logistic regression analysis, including covariates, the baseline number of cigarettes smoked daily in females independently associated with incident RA after a median of 11 (3–18) years ($p=0.002$, Exp β=1.78, 95% CIs 1.24–2.57), but not found in males (Table I). Ranked z-scores of 3 preclinical serum biomarkers also associated independently with incident RA in females, but not in males (Table I). *Lower* values of androstenedione ($p=0.018$), *higher* values of pregnenolone ($p=0.025$) and of sIL-2Rα ($p=0.019$) were associated with incident RA in females, but not in males (Table I).

In males, ranked IgG RF ($p=0.025$, Exp β=1.70, 95% CIs 1.07–2.70) was the only preclinical biomarker independently associated with incident RA (Table I). When entered solely in the models, IgA ($p=0.029$) and IgM ($p=0.024$) isotype RFs significantly associated with incident RA (Table I), but not in combination with IgG.

Identification of the highest rank (*i.e.* 5) of each of the three isotype RFs (IgG, IgA, IgM) revealed that 5 (28%) of 18 RA males had the highest rank of all three isotype RFs *versus* none of 72 CN males ($p<0.001$). Seven of the 18 male incident RA had no highest ranked isotype RF. Comparison of the latter 7 case intervals from 1974 cohort entry to diagnosis of RA was closely similar to the 5 highest ranked isotype RFs male RA cases. The independent associations of preclinical biomarkers with incident RA observed in each sex (Table I), was not significant in the opposite sex (data not shown).

Regarding mortality (Table II), total deaths were greater ($p=0.003$) in the 54 incident RA cases (70%) than in the 216 matched CN (48%) subjects (OR 2.65, 95% CI (1.40–5.05)). Greater RA *versus* CN total mortality occurred in both females (69% *vs*. 49%) and males (72% *vs*. 43%), $p=0.039$ and $p=0.035$, respectively (Table II). The greater total mortality in RA was found in Cox proportionate hazards analyses ($p=0.024$), including covariates (Table II, Fig 1A).

Respiratory-related CODs were greater

Table II. Total and major causes of death (CODs) in incident rheumatoid arthritis (RA) and matched non-RA controls (CN).

A. CODs in All 54 RA and 216 matched CN*

Underlying major causes of deaths	RA (n=54) n (%)	CN (n=216) n (%)	Odds ratios (95% C.I.)	RA *vs*. CN *p*-values
Cardiovascular	12 (22.2)	38 (17.6)	1.34 (0.64-2.78)	0.435
Respiratory	8 (14.8)	11 (5.1)	3.24 (1.23-8.51)	0.017
Neoplasms	8 (14.8)	18 (8.3)	1.81 (0.78-4.67)	0.154
Other, unknown	10 (18.5)	35 (16.2)	1.18 (0.54-2.55)	0.683
Total deaths	38 (70.4)	102 (47.7)	2.65 (1.40-5.05)	0.003

*Deaths occurred between 1984 to 2015, and CODs were not obtained in 10 (4.6%) of CN, but none in RA.

B. CODs in 36 female RA and 144 CN*

Underlying major causes of deaths	RA (n=36) n (%)	CN (n=144) n (%)	Odds ratios (95% C.I.)	RA *vs*. CN *p*-values
Cardiovascular	10 (27.8)	30 (20.8)	1.46 (0.64-3.36)	0.376
Respiratory	6 (16.7)	5 (3.5)	5.56 (1.59-19.40)	0.009
Neoplasms	2 (5.6)	5 (3.5)	1.64 (0.30-8.79)	0.628
Other, unknown	7 (19.4)	31 (21.5)	0.88 (0.35-2.20)	1.000
Total deaths	25 (69.4)	71 (49.3)	2.34 (1.07-5.10)	0.039

*Females had greater observed major CODs in:
40 CVD (22.2%) in 180F *vs*. 10 (11.1%) in 90M, p=0.031.
38 Other and unknown CODs (21.1%) in 180F *vs*. 7 (7.7%) in 90M, p=0.005.

C. CODs in 18 male RA and 72 CN*

Underlying major causes of deaths	RA (n=18) n (%)	CN (n=72) n (%)	Odds ratios (95% C.I.)	RA *vs*. CN *p*-values
Cardiovascular	2 (11.1)	8 (11.1)	1.00 (0.19-5.17)	1.000
Respiratory	2 (11.1)	6 (8.3)	1.37 (0.25-7.46)	0.658
Neoplasms	6 (33.3)	13 (18.1)	2.27 (0.72-7.16)	0.197
Other, unknown	3 (16.7)	4 (5.6)	3.40 (0.69-16.80)	0.140
Total deaths	13 (72.2)	31 (43.1)	3.44 (1.11-10.67)	0.035

*Greater neoplasm related deaths were observed in 19 (21.1%) of 72M *vs*. 7 (3.9%) of 180F, p<0.001.

(p=0.017) in total RA than CN (15% *vs*. 5%) subjects, univariate odds ratio (OR) 3.24, 95% CI (1.23–8.51), but the difference was observed only in females (p=0.009) (Table II). The remaining causes of death did not differ significantly between the study groups in total subjects, females or males (Table II). Proportionate mortality from CVD was greater in females than males, both in RA and CN, contributed by excluded neoplasm deaths in women (see *Methods*) (Table II). Neoplasm-related mortality did not differ between RA *versus* CN subjects (Table II).

The RA *versus* CN status independently associated with the total 140 deaths in all 270 subjects over follow-up from 1995 through 2015 in a Cox regression model (p=0.011, Exp β=1.66, 95% CI 1.12–2.45), including demographic covariates as well as two preclinical ranked z-score biomarkers of hsCRP and sIL-R2α (Table III, Fig 1A). In 1995, 3 categories of course-wide responses to rheumatologist's therapy (Rxresp) were determined (good=19, moderate=23, and limited=12), which independently associated with total mortality in all subjects (p=0.022, Exp β=1.34, 95% CIs 1.04–1.71) and in males (p=0.038), but not in females (p=0.193) (Table III, Fig 1B-C).

Mortality was lower (p=0.19) in the total good (n=19) *versus* moderate (n=23) RA subgroups (Fig 1B-C). In men, the difference between good *versus* limited subgroups was significant (p=0.013), but not in women (p=0.147). History of cigarette smoking was far more prevalent (p<0.001) in 19 good response patients (n=18, 94.7 percent) than in the remaining 35 RA cases (n=16, 45.7%). Males succumbed at an earlier age than females, having mean (±SE) ages at death of 74.5 (±1.14) and 78.7 (±0.99) years, respectively. Percentile deaths before age 80 was greater (p=0.012) in men (72.7) than women (50.0), as may be expected in the general population.

In total subjects, ranked hsCRP values independently (p=0.028) associated with total mortality (Exp β 1.16 (95% CIs 1.01–1.32)), which was significant (p=0.008) in females, but not males (p=0.976), although confidence intervals overlapped (Table III). Rank sIL-2Rα z-scores also associated independently (p=0.030) with total mortality (Exp β 1.15 (95% CIs 1.01–1.30)). The sIL-2Rα biomarker was independently associated with mortality in females (p=0.036), but not in males (p=0.526) (Table III).

Multivariate analysis of respiratory-related mortality revealed significant

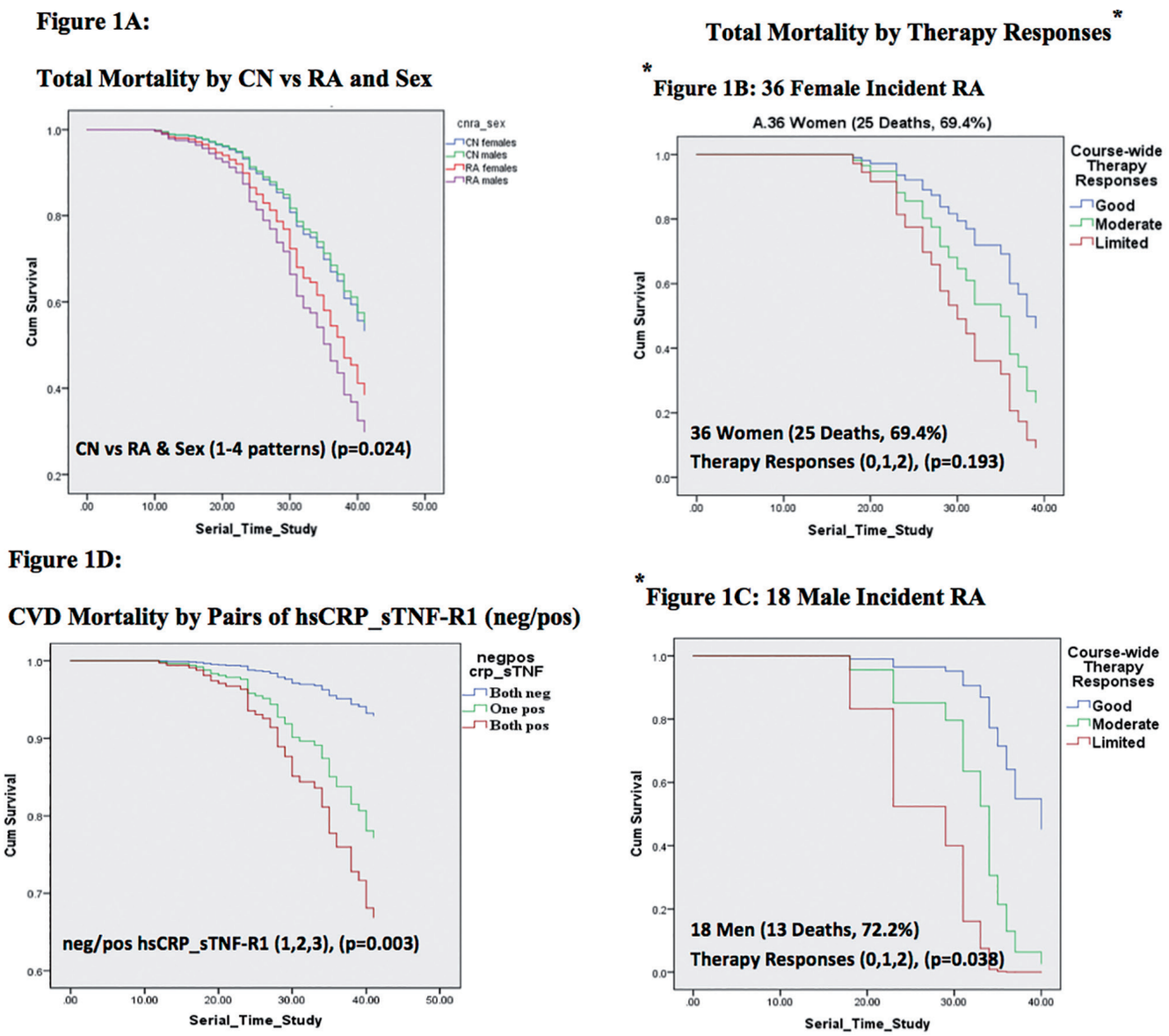

Fig. 1. A-D are all derived from Cox proportionate hazards models (clockwise). **A**: Survival from all causes of death by CN *vs*. RA and sex study groups; **B**: Survival from all causes of death by 3-categories of therapy responses in 36 female incident RA; **C**: Survival from all causes of death by 3-categories of therapy responses in 18 male incident RA; **D**: Survival from CVD deaths in total subjects by combined dichotomies (neg *vs*. pos) of hsCRP and sTNF-R1 (0=both negative, 1=one positive, 2=both positive).

associations with age in total (p=0.001), female (p=0.008), and male (p=0.027) subjects, as well as cigarettes smoked (0–6 scale) in total (p=0.004) and female (p=0.022) subjects (Table IV). Respiratory-related mortality was greater in RA *versus* CN in total (p=0.025, Exp β 3.04, 95% CIs 1.15–8.08) and female (p=0.024, Exp β 4.87, 95% CIs 1.24–19.20) subjects, but not in males (Table IV). Responses to therapy (Rxresp) significantly (p=0.029) associated with respiratory-related mortality in total subjects (Exp β 2.17, 95% CIs 1.08–4.35).

Cardiovascular disease (CVD) mortality did not differ significantly in total, female, or male RA *versus* CN subjects in Cox survival analysis, including demographic and biomarker covariates (Table V). Older age at cohort entry strongly (p<0.001) associated with CVD deaths in total and female subjects, but not in males (p=0.760). Cohort entry age was a significantly (p<0.050) greater predictor of CVD mortality in women (Exp β=3.94, 95% CIs 2.59–6.00) than in men (Exp β = 1.13, 95% CIs 0.52–2.42) (Table V). Median cohort entry age was significantly (p<0.001) older in female CVD decedents (51.0) *versus* all other females (43.0), but not in males (41.0 and 41.5, respectively).

Preclinical ranked serum sTNF-R1 z-scores associated with CVD mortality in total (p=0.003) and female (p=0.010) subjects, but not in males (p=0.126) (Table V). Preclinical ranked serum hsCRP z-scores also predicted CVD mortality in total (p=0.005) and female subjects (p=0.002), but not in males (p=0.889) (Table V). When serum hsCRP and sTNF-R1 were each analysed as continuous z-score values, hsCRP did not quite associate with CVD mortality indepen-

Table III. Baseline Demographic and preclinical serum biomarkers associated with of total mortality to 2015 in total subjects, females, and males.

1974 Demographic and Biomarker Predictors of Mortality to 2015	Total subjects (n=270)		Females (n=180)		Males (n=90)	
	p	Exp β (95% CIs)	*p*	Exp β (95% CIs)	*p*	Exp β (95% CIs)
Entry age (decades)	0.000	2.80 (2.26-3.47)	0.000	3.00 (2.30-3.80)	0.000	2.59 (1.65-4.07)
Sex	0.448	1.17 (0.78-1.75)	N/A	N/A	N/A	N/A
Education completed (yrs)	0.108	0.94 (0.88-1.01)	0.260	0.95 (0.86-1.04)	0.089	0.90 (0.80-1.02)
Cigarettes smoked (0-6 scale)	0.137	1.10 (0.97-1.25	0.180	1.15 (0.94-1.41)	0.292	1.10 (0.92-1.30)
CN *vs*. RA (0 *vs*. 1)	0.011	1.66 (1.12-2.45)	0.173	1.41 (0.86-2.32)	0.032	2.19 (1.07-4.47)
Responses to therapy	0.022	1.34 (1.04-1.71)	0.193	1.25 (0.89-1.75)	0.038	1.49 (1.02-2.16)
Rank of hsCRP z-scores*	0.028	1.16 (1.01-1.32)	0.008	1.25 (1.06-1.47)	0.976	1.00 (0.80-1.25)
Rank of sIL-2Rα z-scores	0.030	1.15 (1.01-1.30)	0.036	1.18 (1.01-1.37)	0.526	1.08 (0.85-1.38)

*Rank of ASAA (n=200) equivalently predicted eoeal deaths, but rank of hsCRP (n=270) was chosen for its assays in all subjects.

Table IV. Baseline demographic and preclinical biomarkers associated with respiratory mortality to 2015 in 270 subjects, 180 females, and 90 males.

1974 Demographic and Biomarker Predictors of Mortality to 2015	Total subjects (n=19)		Females (n=11)		Males (n=8)	
	p	Exp β (95% CIs)	*p*	Exp β (95% CIs)	*p*	Exp β (95% CIs)
Entry age (decades)	0.001	3.00 (1.57-5.71)	0.008	3.05 (1.34-6.92)	0.027	4.14 (1.17-14.60)
Sex (F=0, M=1)	O.597	1.34 (0.46-3.94)	N/A	N/A	N/A	N/A
Education completed (yrs)	0.361	0.92 (0.76-1.11)	0.691	1.06 (0.79-1.43)	0.255	0.86 (0.66-1.12)
Cigarettes smoked (0-6 scale)	0.004	1.55 (1.15-2.09)	0.022	1.76 (1.09-2.84)	0.073	1.40 (0.97-2.02)
CN *vs*. RA (0 *vs*. 1)	0.025	3.04 (1.15-8.08)	0.024	4.87 (1.24-19.20)	0.549	1.70 (0.30-9.58)
Responses to therapy	0.029	2.17 (1.08-4.35)	0.139	2.37 (0.76-7.41)	0.197	1.74 (0.75-4.03)

dently of sTNF-R1 in total subjects (p=0.051) or in females (p=0.061). The 3-category gradient of paired dichotomous z-scores (neg *vs*. pos) of hsCRP and sTNF-R1 biomarkers (0=both negative, 1=either positive, 2=both positive) strongly (p<0.001) predicted CVD mortality in total subjects (Fig. 1D), which was observed in women (p=0.005) but not in men (p=0.187).

Discussion

Preclinical smoking and biomarker associations with incident RA in women (Table I)

Daily cigarettes smoked (0=non-smoker, 6=greater than 50+) strongly (p=0.002) associated with incident RA in women (Table I) consistent with previous reports (1, 3). The current steroid associations of lower ranked serum androstenedione and higher pregnenolone in female incident RA complement our previous report of relative androgenic-anabolic deficiency in preclinical premenopausal women (36).

The higher preclinical ranked z-scores of sIL-2Rα before onset of incident RA in this cohort (Table I) complements a previous, larger-sample report that baseline serum sTNF-R1 was positively associated (p=0.004) with incident RA in women (37). Although complex, an analysis of serum levels of sIL-2Rα and its minor allele in relation to HLA-DRB1 shared epitope (SE) suggest that the sIL-2Rα immunological biomarker may be an independent risk factor for development of RA as well as its radiographic progression and persistence, which deserve further investigation (38).

Independent preclinical biomarker associations with incident RA in men (Table I)

Rank of serum IgG RF was independently associated (p=0.025) with incident RA in men, including other isotype RFs and demographic covariates in the model (Table I). A previous analysis of preclinical IgA and IgM RF levels in this cohort revealed that combined upper-half serum IgA (20+ TU/ml) and IgM (10+ IU/ml) levels associated (p=0.005) with incident RA cases with onsets after 3–20 (median 12) years (data can be provided, *Arthritis Rheum* 2013; 65 (Suppl. 10): S979, abstract 2298). Rheumatoid factor and possibly anti-citrullinated protein antibodies (ACPAs) are considered to be long-term precursors of clinical RA onset, rather than immunological responses to subclinical inflammation (39). The interval from baseline RF testing until clinical onset of RA did not differ between 5 males who had the highest titres *versus* 7 who had the lowest titers, suggesting that this biomarker does not reflect proximity to disease presentation.

Demographic, biomarker and disease severity associations with total mortality (Tables II and III, Figure 1A-B-C)

The degree of response to therapy in 1995 associated with total mortality from 1995 through 2015 in all subjects (p=0.022) and in males (p=0.038) (Table III, Fig 1C). Ranked serum hsCRP and sIL-2Rα preclinical biomarkers independently associated with incident RA in all subjects and in females, but not males (Table III).

Male RA cases were reported to die at a younger age than females (23, 40). In one report, women were considered

Table V. Baseline (1974) demographic and preclinical serum biomarkers associated with cardiovascular deaths to 2015 in 270 total, 180 female, and 90 male subjects. of total mortality to 2015 in total subjects, females.

1974 Demographic and Biomarker Predictors of Mortality to 2015	Total subjects (n=270)		Females (n=180)		Males (n=90)	
	p	Exp β (95% CIs)	*p*	Exp β (95% CIs)	*p*	Exp β (95% CIs)
Cohort entry age (decades)*	0.000	3.07 (2.14-4.40)	0.000	3.94 (2.59-6.00)*	0.760	1.13 (0.52-2.42)*
Sex (F=0, M=1)	0.340	0.69 (0.33-1.47)	NA	NA	NA	NA
Completed education (yrs)	0.450	0.96 (0.85-1.08)	0.609	0.96 (0.84-1.11)	0.266	0.86 (0.66-1.24)
Cigarettes smoked (0-6 scale)	0.916	0.99 (0.77-1.26)	0.694	1.07 (0.76-1.52)	0.587	0.89 (0.60-1.34)
CN *vs*. RA (0 *vs*. 1)	0.148	1.66 (0.34-3.28)	0.265	1.55 (0.72-3.37)	0.691	1.40 (0.27-7.23)
Responses to therapy	0.737	1.08 (0.70-1.66)	0.499	0.84 (0.50-1.41)	0.099	1.92 (0.88-4.19)
Rank of sTNF-R1†	0.003	1.39 (1.12-1.72)	0.010	1.37 (1.08-1.74)	0.126	1.52 (0.89-2.60)
Rank of hsCRP‡	0.005	1.39 (1.11-1.75)	0.002	1.52 (1.16-1.98)	0.889	1.03 (0.64-1.67)

*$p<0.050$, cohort entry-age is a greater predictor of CVD mortality in women (HR 3.94, 95% CI 2.59-6.00) *vs* men (HR 1.13, 95% CI 0.52-2.42).
†Rank of TNF-α was also a predictor of CVD deaths, when alone in the model, but not independently of rank of sTNF-R1, and not included.
‡Rank of ASAA (n=200) equivalently predicted CVD deaths, but rank of hsCRP (n=270) was chosen for its assays in all subjects.

to have a more aggressive disease and a poorer long-term outcome than men (41), not confirmed in 2 other reports (20, 42) or in our results (Tables II, III, Fig 1A). Both sexes showed comparable mortality trends by categories of course-wide therapy responses, with greater mortality among RA patients having only moderate and limited therapy responses (Fig 1B-C), consistent with previous reports (20, 43).

Increased respiratory-related mortality in incident RA patients (Tables II and IV)

Greater respiratory-related deaths were observed in female RA *versus* CN subjects in this cohort, but not in male counterparts (Tables II, IV). Cigarette smoking significantly predicted respiratory-related CODs in all subjects (p=0.004), females (p=0.022), and nearly so in males (p=0.073) in Cox regression models, including demographic covariates (Table IV). Responses to therapy (Rxresp) was associated with respiratory-related mortality in total subjects (Table IV).

In a recent long-term Nurses' Health Study (NHS) (44), respiratory-related cause mortality was increased in women who developed incident RA, compared to women without this disease (HR, 2.06; 95% CI, 1.51–2.80). A recent longitudinal observational study of US veterans with RA also found a 3-fold increased risk of respiratory-related death in RA cases, mainly males, compared to the general population expectation (45).

Biomarkers associated with cardiovascular disease (CVD) mortality (Table V, Fig 1D)

Cohort entry age was not significantly associated with CVD mortality in males (p=0.760), as opposed to females ($p<0.001$) (Table V). Males have a younger age of CVD deaths than females in the population (46) and in a nation-wide study of persons with inception RA (23). Ranked values of preclinical serum hsCRP and sTNF-R1 independently associated with CVD mortality in this cohort (Table V, Fig 1D). Throughout this study, no contradiction of set rank vs continuous biomarker z-score values was found in their associations with incident RA or mortality. Pairing of dichotomous (neg *vs*. pos) hsCRP and sTNF-R1 preclinical values strengthened ($p<0.001$) the prediction of CVD mortality in total subjects over each ranked value alone (Table V, Fig 1D). Ranked hsCRP was a greater predictor of total and CVD mortality in females than in males (Tables III and V), which may suggest a possible sexual dimorphism in inflammation pathways (27).

Increasing attention is being given to the preclinical phase of RA with focus on possible prevention of disease and improved outcome (47). Considerable research has been devoted to serum hsCRP as a risk predictor of coronary heart disease (CHD) and related CVD mortality (48). However, the complexity of synthesising heterogeneous studies and measuring different genetic variants confound interpretation of a single causal estimate (49, 50). The latter considerations may minimise a causal effect of hsCRP on CHD (49, 50). Criteria for evaluation of novel biomarkers of cardiovascular or other disease risk depends on clinical value and effect on patient management and outcomes, and even on cost-effectiveness (51). Additional studies combining baseline biomarkers (52) may improve their risk stratification. We did not pursue the predictive ability of serum ASAA in this study, since that value was missing in 70 females, but it has also been reported to correlate with differential response to various therapies in early rheumatoid arthritis and may have additional predictive value as a disease activity biomarker (53).

A main limitation of this observational study is the modest sample sizes of incident RA cases and matched CN subjects as well as the single-time assessment of the baseline pre-clinical demographic and biomarker variables in 1974. Other reservations of our findings include: 1) inability to categorise course patterns of RA patients on biological drug therapy; 2) exclusion of most female (RA and CN) subjects who had known cancer-related diagnoses by 1992; 3) a limited number (4.6 percent) of CN subjects with unknown CODs due to having moved out of state; 4) unavailability of data on serum ACPAs and HLA-DR epitopes; 5) an increased risk of Type 1 error from interpreting the many NEI biomarker analyses related to incident RA and mortality predictors, and 6) potential

bias due to unobserved confounders in this observational study.

Strengths of this nested case-control study design include: 1) a 41-year defined community-based cohort sample; 2) assays of multiple 1974 baseline preclinical NEI biomarker levels in RA and CN subjects; 3) the sole community rheumatologist's assessment in 1995 of RA patients' overall course responses to therapies, and 4) long-term mortality follow-up of study subjects, from 1995 through 2015. The positive findings in this study support the reported significant excess of total and respiratory-related mortality in RA *versus* CN subjects. The rheumatologist's assessment of incident RA patients' therapy responses in 1995 also supports the reported increased mortality hazard of greater disease severity manifestations (20, 40). Novel preclinical serum neuroendocrine and immune (NEI) biomarkers were identified which associated with incident RA and its subsequent mortality from all-causes and from CVD.

We endorse the recommendation of the recent survey on systematic reviews (24) that further subgroup analysis or individual patient data meta-analysis would provide more specific results on sex effects from reported literature than unqualified systematic reviews. The current long-term prospective study has been costly in finance and investigator efforts and results would be enhanced by mechanisms and programs that would allow sharing of data from such cohort studies as well as systematic reviews (24), and clinical trials (54, 55). However, the concept of data sharing and its most effective implementation is a topic of debate (56, 57).

In conclusion, this long-term community-based cohort study confirmed an increased total and respiratory-related mortality of incident RA patients than matched CN subjects. Increased mortality occurred only in those cases who had less than a good pattern of therapeutic response to rheumatologist's management. Preclinical serum biomarkers were identified which associated with incident RA as well as its subsequent total and CVD mortality. The preceding biomarker associations with mortality were significant in females, not males, and deserve to be assessed in independent and larger sample population studies.

Acknowledgements

Subjects' baseline entry demographic data and preclinical sera were provided by The CLUE Study, Campaign Against Cancer and Stroke, funded in 1974 by Grant CA 118949 from the National Cancer Institute.

Dr Raymond L. Malamet, the sole community rheumatologist, provided invaluable assistance in identifying incident RA cases in 1992 and 1994, and evaluated each of his RA patients for course patterns of therapy response, in 1995.

Mortality follow-up to 2015 was carried out by Judith Hoffman-Bolton.

Steroidal and hormonal assays were performed by Dr Robert T. Chatterton in the laboratory of Northwestern University (NWU), Feinberg School of Medicine, Chicago, IL.

Acute phase proteins, ASAA and hsCRP, were performed by Dr Jean D. Sipe (retired) in the laboratory of Department of Biochemistry, Boston University School of Medicine, Boston.

Multiple imputations were performed by Dr Huaping Wang of UICOMP of randomly missing values.

Isotype RF assays were performed by Dr M.C. Teodorescu at TheraTest Laboratories, Inc., Lombard, IL.

Particular appreciation is expressed for Emily Walsh, who had contributed extensively in the analyses and preparation of tables and figures.

This project was supported by the Dept. of Medicine, University of Illinois College of Medicine at Peoria and by a gift from the MTM Foundation.

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