Immunoradiometric Assay of Circulating C-Reactive Protein: Age-related Values in the Adult General Population

WINSTON L. HUTCHINSON,[1*] WOLFGANG KOENIG,[2] MARGIT FRÖHLICH,[2] MALTE SUND,[3] GORDON D.O. LOWE,[4] and MARK B. PEPYS[1*†]

Background: **Increased values of C-reactive protein (CRP), the classical acute phase protein, within the range below 5 mg/L, previously considered to be within the reference interval, are strongly associated with increased risk of atherothrombotic events, and are clinically significant in osteoarthritis and neonatal infection.**
Methods: **A robust new polyclonal-monoclonal solid-phase IRMA for CRP was developed, with a range of 0.05–10.0 mg/L.**
Results: **Plasma CRP values in general adult populations from Augsburg, Germany (2291 males and 2203 females; ages, 25–74 years) and Glasgow, Scotland (604 males and 650 females; ages, 25–64 years) were very similar. The median CRP approximately doubled with age, from ~1 mg/L in the youngest decade to ~2 mg/L in the oldest, and tended to be higher in females.**
Conclusion: **This extensive data set, the largest such study of CRP, provides valuable reference information for future clinical and epidemiological investigations.**
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[1] Immunological Medicine Unit, Division of Medicine, Imperial College School of Medicine, Hammersmith Hospital, London W12 0NN, United Kingdom.
[2] Department of Internal Medicine II-Cardiology, University of Ulm Medical Center, D-89081 Ulm, Germany.
[3] GSF-National Research Center for Environment and Health, MEDIS-Institute, D-85764 Neuherberg, Germany.
[4] Department of Medicine, Royal Infirmary, Glasgow G312ER, United Kingdom.
*Current address: Centre for Amyloidosis and Acute Phase Proteins, Department of Medicine, Royal Free and University College Medical School, London NW3 2PF, United Kingdom.
†Address correspondence to this author at: Department of Medicine, Royal Free and University College Medical School, Rowland Hill St., London NW3 2PF, United Kingdom. Fax 44-20-7433-2803; e-mail m.pepys@rfc.ucl.ac.uk.
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Measurement of C-reactive protein (CRP),[5] the classical acute phase protein, is an extremely valuable marker of disease activity and response to therapy in a wide range of tissue-damaging, inflammatory, infective, and neoplastic conditions *(1)*. The original automated nephelometric and turbidimetric immunoassays for CRP developed in the 1970s and the homogeneous enzyme and fluorescence polarization methods introduced in the early 1980s had limited sensitivity and poor precision in the low range. Thus, values below 5–10 mg/L could not be detected, although later improvements in some systems reduced the limit of detection to ~2 mg/L. This was not a problem because the median value for serum CRP in apparently healthy adults is ~0.8 mg/L, the 90th centile of the distribution in such subjects is ~3 mg/L, and all the information required for the classical applications of clinical CRP measurement in adults derives from increases in CRP concentration above these concentrations *(1, 2)*. However, the recent use of more sensitive immunoassays for CRP has revealed exciting new information of both fundamental and clinical importance in three different areas: coronary heart disease and other complications of atherosclerosis *(3–15)*, osteoarthritis *(16)*, and neonatal infections *(17)*.

Although there now are several commercially available, routine clinical chemistry assays capable of precisely measuring circulating CRP concentrations within what was previously considered to be the reference interval, these were not available when we commenced our studies *(3, 6)*. We therefore developed, and report here, a sensitive solid-phase monoclonal-polyclonal IRMA designed

to measure CRP in plasma or serum of all subjects in the general population *(12, 18–21)*.

Materials and Methods

CRP AND ANTI-CRP ANTIBODIES

CRP was isolated and purified as described previously *(22, 23)* and was used to raise monoclonal mouse and monospecific polyclonal goat anti-human CRP antisera. Mouse monoclonal anti-CRP antibodies were isolated from ascitic fluid by sodium sulfate precipitation and DEAE-Sepharose ion-exchange chromatography. Goat polyclonal anti-CRP antibodies were isolated by thiophilic affinity chromatography on a broad spectrum immunoglobulin-binding matrix (T-Gel; Bioaffinity Systems).

IMMOBILIZATION OF POLYCLONAL ANTI-CRP

Microtiter plates with *N*-oxysuccinimide-activated surfaces (Corning Costar) were coated with goat anti-CRP antibodies by incubation with 100 μL per well of the immunoglobulin fraction at 100 mg/L in phosphate-buffered saline (PBS), pH 9.0, for 1 h at 21 °C. After decanting, the wells were each washed twice with 200 μL of PBS, pH 7.4, containing 0.5 mL/L Tween 20 (Bio-Rad Laboratories), before blocking for 30 min with 100 μL per well of 20 g/L bovine serum albumin in PBS, pH 7.4. After each well underwent a final rinse step (three times with 200-μL volumes of PBS), the plates were used for assays on the same day.

RADIOLABELING OF MONOCLONAL ANTI-CRP

Isolated monoclonal anti-CRP antibodies were oxidatively iodinated using *N*-bromosuccinimide *(24)* and carrier-free Na^{125}I, and after separation by gel filtration on G25 Sephadex (PD10 column; Pharmacia Biotech AB), the labeled antibodies had a typical specific activity of 17 kBq/μg.

IRMA FOR CRP

Calibrators were constructed that contained isolated CRP at concentrations of 0.05, 0.10, 0.25, 0.50, 1.00, 5.00, and 10.00 mg/L in solution in 0.14 mol/L NaCl, 0.01 mol/L Tris, 0.002 mol/L CaCl$_2$ (pH 8.0), containing 10 g/L bovine serum albumin and 2 mL/L Tween 20 (TCBT buffer). These calibrators, together with serum or plasma samples for assay and control sera with known CRP concentrations, were each diluted 1:100 in the same TCBT buffer but containing CaCl$_2$ at a final concentration of 0.01 mol/L, and were then loaded in triplicate into the anti-CRP-coated plates at 100 μL per well. The additional calcium was included to ensure availability of free calcium ions in all samples including EDTA plasma. After incubation at 37 °C for 1 h, the plates were decanted, and each well was washed three times with 200 μL of TCBT buffer. Captured CRP was then detected by addition to each well of 100 μL of ^{125}I-labeled monoclonal anti-CRP antibody containing an activity of 100 000 cpm. After

incubation at 37 °C for 1 h, the plates were decanted, and each well was washed three times with 200 μL of TCBT buffer before finally being blotted dry and counted individually in the gamma counter. The calibration curve was constructed using a four-parameter logistic curve-fit program, and values for samples and controls were determined by interpolation. All samples with values at the top of the assay range, i.e., $\geq$10 mg/L, were reassayed at appropriately higher sample dilutions.

ASSAY VALIDATION

Intra- and interassay CVs were initially determined in London using sera from four patients (three pregnant females and one male with reactive systemic AA amyloidosis). Each sample was assayed in quadruplicate in each of three separate assays conducted within a period of 21 days. Recovery of CRP was tested by adding known amounts of pure CRP into two separate clinical samples and then assaying them in quadruplicate in three separate assays. We looked for correlation between the present method and an established high-sensitivity CRP immunoassay, using 80 serum samples from healthy adult laboratory staff in whom values had been determined previously by the method we developed on the Abbott IMx® instrument (Abbott Laboratories) *(25)*. Results were compared by the Bland-Altman method to establish the mean difference. Subsequently, the assay was transferred to Ulm where intra- and interassay CVs were determined as in London. The results were the same, and the calibration curves were superimposable and were extended to confirm complete concordance between standardization on the purified CRP calibrators and using the WHO International Reference Standard for CRP Immunoassay, 85/506 *(26)*. All of the population samples described below, as well as the samples in our other published reports *(12, 18–21)*, were assayed in Ulm. A control plasma pool created at the start of the study and used in every assay run in Ulm, gave essentially identical results over a 3-year period. No differences were observed between serum and plasma samples taken from the same venesection.

CLINICAL SAMPLES

Plasma samples from 2291 males and 2203 females, ages 25–74 years, collected during 1994–1995 in Augsburg, Germany, and from 604 males and 650 females, ages 25–64 years, collected during 1994 in Glasgow, Scotland were assayed in the present IRMA to establish CRP values in the adult general population. The Augsburg participants were selected from the general population using a two-stage, age- and sex-stratified random cluster sampling method. The Glasgow subjects were randomly selected from general practitioners' lists in North Glasgow District. All subjects gave informed consent, and all protocols and collection procedures were approved by the relevant institutional committees. Nonfasting blood was collected into Na$_2$/Na$_4$EDTA from the antecubital vein, in

the sitting position with minimal suction and short-term occlusion. Plasma was obtained by centrifugation at 3000g for 15 min and was stored immediately at −70 °C until used for analysis. All assays were performed in Ulm in a single continuous batch. The main and interactive effects of age, sex, and location were sought by classical ANOVA techniques for unbalanced data, using $\log_n$ transformation of CRP values, which greatly improved compliance with normal theory assumptions. All computations were performed on a personal computer in Windows NT 4 with SAS software, Release 6.12.

Results

ESTABLISHMENT AND VALIDATION OF THE ASSAY

The calibrators yielded a response proportional to the concentration used between 0.05 and 10 mg/L. There was no overlap in readings between the 0.05 mg/L calibrator and the zero control consisting of TCBT buffer alone without added CRP ($P = 0.01$, t-test). The difference between the mean readings for 0.05 mg/L and for the zero control exceeded 3 SD of either mean in all assays, and 0.05 mg/L was thus the minimum detectable concentration in this assay. Readings reached a plateau for samples containing >10 mg/L, and these were therefore re-assayed at higher dilution.

The intraassay CVs ranged from 5.6% for the 0.50 mg/L calibrator to 1.4% for the 0.10 mg/L calibrator. The interassay CVs ranged between 8.0% for the 5.0 mg/L calibrator and 0.5% for the 0.10 mg/L calibrator. Testing in quadruplicate, in three separate assays on 3 different days, of clinical samples with values between 0.10 and 4.40 mg/L in the IRMA and 0.10 and 4.20 mg/L in the IMx assay gave intraassay CVs of 1.8−6.2% and interassay CVs of 2.8–11%. When pure CRP was added to two clinical samples containing 0.10 and 0.60 mg/L, respectively, to increase their concentrations to 0.60 and 1.10 mg/L, respectively, re-assaying yielded mean (SD) recoveries of 93% (9.6%) and 101% *(12)*.

Sera from 80 apparently healthy adults measured in the IMx assay and by the IRMA had values of 0.05–8.40 mg/L and 0.05–8.20 mg/L, respectively. Bland-Altman comparison after log transformation of both data sets gave a mean difference, expressed as the ratio IRMA/IMx, of 1.04, with 95% limits of agreement of 0.54 and 2.00, indicating excellent agreement between the two methods.

CRP VALUES IN THE GENERAL POPULATION

The results from this extremely large survey (Table 1) were comparable to those reported previously, with the distribution very heavily skewed to the right, the median close to 1 mg/L, and only ~5% of samples containing >10 mg/L.

There was a significant trend to higher CRP values with increasing age in both locations. ANOVA on $\log_n$ CRP for effect of age gave $P <0.0001$ for both Augsburg (ages, 25–74 years) and Glasgow (ages, 25–64 years). For the Glasgow results, ANOVA on $\log_n$ CRP was not significant for either the effect of sex ($P = 0.5248$) or for the interaction of age and sex ($P = 0.3864$). However, for the Augsburg results, ANOVA on $\log_n$ CRP for the effect of sex was significant, with females being marginally

Table 1. Distribution of plasma CRP values in the adult general population.

Plasma CRP concentration, mg/L

Location	Sex	Age, years	n	Geometric mean	SD	Mean	Minimum	Percentile								Maximum
								1st	2.5th	5th	25th	75th	95th	97.5th	99th	
Augsburg	M	25–34	433	0.81	3.07	0.75	0.05	0.05	0.08	0.12	0.40	1.70	4.88	7.27	10.72	80.69
		35–44	435	1.12	3.10	1.13	0.05	0.07	0.11	0.17	0.54	2.39	6.29	9.16	15.46	112.47
		45–54	467	1.40	2.97	1.31	0.07	0.15	0.19	0.28	0.65	2.91	8.51	13.95	18.90	74.53
		55–64	492	1.56	2.74	1.50	0.06	0.15	0.23	0.32	0.79	3.01	8.16	12.22	22.25	36.48
		65–74	464	2.03	2.76	1.93	0.05	0.23	0.33	0.38	1.06	3.88	9.44	18.47	30.60	46.06
	F	25–34	412	1.08	3.77	1.02	0.05	0.05	0.07	0.11	0.48	2.45	8.49	17.18	37.22	49.75
		35–44	475	1.13	3.47	1.08	0.05	0.06	0.10	0.17	0.46	2.66	8.57	14.86	22.29	34.64
		45–54	477	1.29	3.14	1.33	0.08	0.11	0.15	0.20	0.56	2.85	7.92	12.12	25.06	92.81
		55–64	470	1.83	2.75	1.76	0.10	0.15	0.25	0.35	0.98	3.66	8.97	10.97	25.34	78.19
		65–74	369	2.18	2.66	2.22	0.05	0.21	0.30	0.43	1.20	4.05	9.86	16.58	27.84	52.19
Glasgow	M	25–34	144	1.01	2.97	1.01	0.06	0.18	0.20	0.25	0.42	2.14	5.93	8.36	19.50	25.44
		35–44	158	1.18	3.24	0.96	0.05	0.06	0.10	0.17	0.58	2.59	7.36	15.78	23.85	25.86
		45–54	147	1.54	2.70	1.34	0.15	0.24	0.28	0.36	0.78	2.89	7.82	21.81	31.01	32.26
		55–64	155	2.14	2.81	1.96	0.27	0.28	0.29	0.38	1.07	4.33	10.59	20.01	23.55	39.97
	F	25–34	177	1.25	3.26	1.33	0.05	0.11	0.12	0.19	0.55	3.23	7.75	10.03	20.34	30.21
		35–44	162	1.12	3.10	0.95	0.08	0.12	0.16	0.22	0.49	2.79	7.34	8.95	13.54	20.13
		45–54	153	1.46	3.24	1.53	0.09	0.13	0.17	0.22	0.68	3.24	8.85	15.47	18.03	20.75
		55–64	158	2.25	3.26	2.40	0.05	0.08	0.15	0.31	1.02	5.27	15.50	24.26	36.31	76.48

higher ($P = 0.0067$), and the interaction of age and sex was also significant ($P = 0.0084$), which complicates interpretation of the main effects. To compare CRP values in the different locations, the Augsburg 65–74 age group was eliminated, and full factorial ANOVA of location-sex-age gave nonsignificant three-factor and two-factor interactions that included location. This permitted interpretation of the main location effect, which was significant ($P <0.0001$) and corresponded to a Glasgow:Augsburg ratio of 1.16:1 on the back-transformed scale.

Discussion

The present IRMA for CRP, performed in two different laboratories in different countries and with several different operators, gave the same ranges of intra- and interassay CVs for all of the calibrators and consistently yielded the same value for a control plasma pool over a period of 3 years. It was sufficiently sensitive to detect CRP in all clinical samples tested and has already provided important new clinical and scientific information (12, 18–21). Results obtained by the IRMA for ostensibly healthy subjects closely matched those from the solid-phase microparticle-enhanced enzyme immunoassay that we developed on the Abbott IMx instrument (25), and those in turn were the same as in our early radioimmunoassay study of CRP concentrations in sera from 469 volunteer blood donors (2). The assays were all standardized on the same highly purified human CRP preparations that we used to construct the WHO International Reference Standard for CRP Immunoassay (85/506) (26) and provided for IFCC Certified Reference Material 470 (27). The expected close agreement of results confirms the specificity and precision of these methods.

The present study of individuals from the general population, all measured in a single continuous batch in a single laboratory, is by far the largest yet reported and provides robust reference information as well as showing several features of interest. The usual distribution of CRP values was found, heavily skewed to the right, and was similar in men and women and in the Glasgow and Augsburg populations, although the Glasgow concentrations were slightly higher than those in Augsburg and the German women had marginally higher concentrations than the German men. The sex difference may reflect the estrogen effect we have reported elsewhere (21).

The median values, ranging from 0.75 to 2.40 mg/L in different age groups, were with just one exception higher than the 0.8 mg/L we reported in our early study of healthy volunteer blood donors (2). The 90th, 95th, and 99th percentiles were also higher. This probably reflects the existence of more subclinical disease in the general population than among those accepted as donors by the United Kingdom National Blood Transfusion Service. Similar higher CRP values have been reported in previous, smaller studies of general populations [for example, see Refs. (13, 15)]. We currently have no explanation for the higher values found in females in Augsburg but not in

Glasgow, or for the higher values in Glasgow than in Augsburg, but it is unlikely to result from sample handling. All samples were collected and stored identically. Using a variety of different in-house (2, 17, 25, 28) and commercial assays (Abbott, Beckman, Technicon, and Roche), we have periodically remeasured CRP concentrations in aliquots of normal serum, acute phase serum, normal and acute phase plasma, and normal serum supplemented with various concentrations of purified CRP that have been stored frozen at −70 °C for >20 years, and found no significant change with time (M.B. Pepys, personal observation). The notable stability of CRP in serum was also documented in our preparation of WHO Standard 85/506 (26).

Although no specific exclusions were imposed in the present study, subjects with known acute active disease were presumably not bled. However, the top 10% or so of CRP values reflect ongoing acute phase responses at the time of sampling, presumably resulting from chronic or low-grade intercurrent infection, inflammation, or other tissue-damaging processes. The increasing prevalence of clinical and subclinical diseases that induce an acute phase response may also underlie the modest, although statistically significant, increase in median CRP concentrations over the decades of age sampled in the present large number of subjects.

Other possible influences on the relationship between age and plasma CRP concentration are smoking and obesity (13). CRP concentrations are known to be affected by smoking, presumably through its inflammatory and tissue-damaging effects and the smoker's increased susceptibility to respiratory infection. Obesity is associated with increased CRP concentrations, presumably because adipose tissue is an important site for production of interleukin-6, the major up-regulator of CRP gene expression (29). CRP values are also associated with components of the insulin-resistance syndrome (30); however, at present, we lack the information required to investigate such associations in the populations studied here.

The strongly predictive association between increased CRP concentrations and coronary heart disease, ischemic stroke, and peripheral arterial disease is of particular interest and importance (3–13). It has emerged from studies in which individuals known to have diseases capable of inducing an acute phase response have been excluded, and it is observed even at very modestly increased CRP values. However, it is not known whether the increased CRP production reflects the extent of atherosclerosis itself and inflammatory activity within the plaques, inflammation elsewhere in the body, or just genetically determined higher baseline CRP concentrations (31). It is also not known whether CRP itself contributes to the pathogenesis of atheroma and its thrombotic complications or is just a sensitive marker for underlying inflammation and/or tissue-damaging processes that promote atherothrombosis by other mechanisms. These questions will probably be answered only by

studies with drugs capable of specifically inhibiting the production or binding and effects of CRP, and such agents have yet to be developed (32). Nevertheless, if increased CRP production is triggered by the presence, severity, and extent of atheroma, then these factors may contribute to the age-related increase in CRP values.

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