Chapter 4

Analytical evaluation and determination of reference values of soluble interleukin-2-receptor and serum amyloid-A

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Abstract

Background

There is a growing interest in both, serum amyloid A (SAA) as an acute phase reactant and soluble IL-2 receptor (sIL-2R) as a T cell activation parameter. In this study, we evaluated the analytical performance of SAA and sIL-2R assays and we report the reference values in an adequate reference population.

Materials and methods

SAA was determined by particle-enhanced immunonephelometry on the BN Prospec from Dade Behring, whereas sIL-2R was determined on the IMMULITE Automated Analyzer, by means of a two-site chemiluminescent enzyme immunometric assay. Reference intervals were established in 282 adult blood donors

Results

For sIL-2R the coefficients of variation (CV) for within- (n = 20) and between-run (n = 20) assay precision were < 3.8% and < 7.3%, respectively, and for the SAA < 9.2% and < 10.8%. Both methods were linear, sIL-2R below 1000 kU/L and SAA below 854 mg/L. For sIL2R reference intervals were found to be 241 – 846 kU/L (2.5^{th} – 97.5^{th} percentile). For SAA sex differences were observed and the reference values in males were 0.99 – 9.87 mg/L and in females 0.84 – 11.4 mg/L. The sIL-2R concentration appeared not related to age. However, SAA concentrations tended to a moderate increase with age.

Conclusions

Both assays, SAA on the BNProSpec and sIL-2R of the IMMULITE appeared to be useful in clinical practice.

Introduction

Serum amyloid A (SAA) is an acute phase protein. The liver is mainly responsible for the production upon stimulation by interleukins IL-1 and IL-6, respectively. Like C-reactive protein (CRP), SAA belongs to the quick responding acute phase proteins.
The diagnostic value of these two different acute phase proteins is influenced by the underlying disorder. For example, during viral infections, SAA appeared to increase more intense as compared to CRP[1-3]. In some diseases, such as rheumatoid arthritis, Crohn's disease and ulcerative colitis, CRP remained even normal, whereas SAA increased[4-8]. Increased CRP was shown to be associated with inflammation in sarcoidosis[9], whereas SAA appeared to be increased in patients with active sarcoidosis[10]. To date, one of the strongest advantages of SAA above CRP has been demonstrated in kidney transplantation patients on immunosuppressive steroid therapy. In this later population only SAA, but not CRP appeared to be useful in monitoring acute rejection reactions [2,11,12].
Another inflammatory parameter of clinical importance is soluble interleukin-2-receptor (sIL-2R). Interleukin-2 stimulates activation of T cells through a complex interleukin-2 receptor molecule[13]. It concerns a heterodimeric receptor, which is partly released as a soluble form of the 55 kD chain, the so called soluble IL-2 receptor[14,15]. sIL-2R was found to be a good prognostic marker, related to pulmonary as well as extrapulmonary manifestations of sarcoidosis[16-19]. Recently, it has been demonstrated by various groups that sIL-2R appeared to be more useful as compared to the traditionally used angiotensin-converting enzyme and might even replace it[18-20].
Before an assay can be used for clinical evaluation, a critical examination of assay performance is required. In this paper we report an extensive evaluation of the new SAA assay on the BNProSpec, from Dade Behring. Although evaluation of sIL-2R on the IMMULITE is available, no reference values were reported until now[21]. Therefore, the reference values estimated in a population of healthy blood donors for both, SAA, determined on the BN ProSpec from Dade Behring, and for sIL-2R, determined on the IMMULITE are presented.

Materials and methods

Study population

To allow determination of reference values, between 1999 – 2002 venous blood samples were collected from 282 ostensibly healthy blood donors presenting at the Sanguine Blood Bank in Maastricht. The Medical Ethical Committee of the Hospital approved the procedure followed.

Serological measurements

Samples were obtained by venipuncture in serum tubes and clotted blood was centrifuged (2500 g at 20°C) for 15 minutes to obtain serum. Serum was separated and stored at -70°C for further analysis. The whole procedure took less than an hour.
Soluble IL-2 receptor (sIL-2R) was determined on the IMMULITE Automated Analyzer, by means of a two-site chemiluminescent enzyme immunometric assay, with a detection limit of 50 kU/L, and a measuring range 50 – 7500 kU/L (Diagnostic Product Corporation, Los Angeles, CA, cat no LKIP1). IL-2R reagent (LIP2) consists of alkaline phosphatase conjugated to rabbit polyclonal anti-IL-2R antibody.
Serum amyloid A (SAA) was determined on the BN Prospec from Dade Behring, by particle-enhanced immunonephelometry. The detection limit for SAA was 3 mg/L, with a measuring range of 3 – 1000 mg/L, depending on dilution (Dade Behring, Liederbach Germany, N SAA reagent, cat no OQMP 11). N SAA reagent (OQMP 11) consists of lyophilised polystyrene particles coated with sheep antibodies to human SAA.
For both assays, within-run precision was obtained by measuring one sample 20 times within a single run. Between-run precision was obtained by measuring each level on 20 consecutive days, based on a single calibration.
For sIL-2R, samples for precision studies were prepared from two serum pools in the range from 200 – 1000 mg/L. The linearity studies were performed with one sample containing about 1000 kU/L of sIL-2R. Using the manufacturer's diluent (IL-2R Sample Diluent (LIPZ)) eleven dilutions of each sample were made in two replicates and measured.
With respect to SAA, samples for precision studies were prepared from nine serum pools in the range from 0.98 – 821 mg/L, as determined on the BN ProSpec. The linearity studies were performed with two samples containing about 854 mg/L and 150 mg/L SAA. Using the manufacturer's diluent (N Diluent (OUM T65)) nine dilutions of each sample were made in five replicates and measured.

Statistical analysis

Statistical analysis was performed using the SPSS10.0 for Windows (SPSS, Chicago, IL, USA). The distributions of the SAA and sIL-2R were positively skewed. Therefore, the reference values are presented as an interpercentile interval (2.5^{th} – 97.5^{th} range). Linear regression was used to test the relationship with age. Sex-differences were tested by means of Mann-Whitney U test. All p-values were two-tailed, and $p < 0.05$ was considered statistically significant.

Results

Imprecision and linearity of sIL2R assay

In two pools with mean concentrations of sIL-2R of 193 and 978 kU/L between run variations of respectively 3.6 and 7.2% and within run variations of respectively 2.7 and 3.7% were found. The IMMULITE sIL-2R assay appeared to be linear below 1000 kU/L. The results of the regression analysis are presented in Figure 1A. The

percentage deviation of the observed values as compared to expected values varied from -2.3% – 13.6% (mean: 4.8%).

Imprecision and linearity of SAA assay

Since to the best of our knowledge this report concerns, the first evaluation of the SAA assay on the BN ProSpec, slightly more extensive evaluation was performed as compared to the sIL-2R evaluation. For nine pools with mean concentrations of 0.98, 4.31, 7.40, 14.7, 21.64, 85.44, 134, 452 and 821 mg/L between run variations of respectively 10.7, 9.3, 4.4, 4.3, 4.1, 4.8, 4.7, 6.9 and 6.6% and within run variations of respectively 8.9, 9.2, 5.1, 6.3, 2.6, 3.3, 7.4, 5.0 and 5.2% were found. So, the total imprecision for the SAA BN ProSpec method remained below 10.7%. The method appeared to be linear below 854 mg/L. Linearity results are presented in Figure 4.1B. The percentage deviation of the observed values as compared to expected values varied from -21.8% – 17.6% (mean: 2.4%).

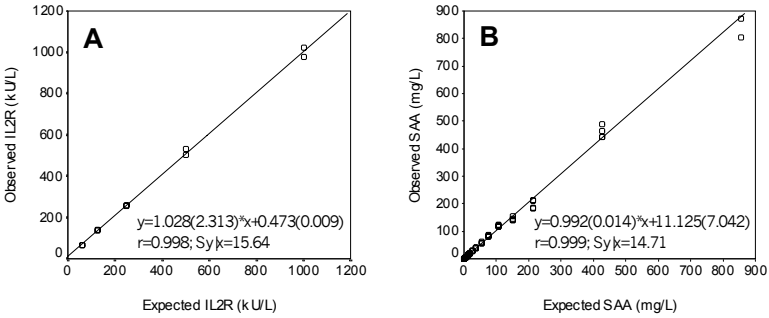

Figure 4.1. Linearity of sIL-2R (IMMULITE) and SAA (BN ProSpec) assays.
The values for each dilution point are plotted vs. expected values and linear regression was performed. Linearity results of sIL-2R IMMULITE (A) and SAA BN ProSpec (B) method are presented for the whole tested range. For sIL-2R, dilution samples were run in duplicate in 2 independent experiments and the results of the one of these experiments are plotted, whereas the SAA linearity was determined in a single experiment in four replicates (SAA). The linearity equation is presented with the standard errors for the intercept and the slope within parentheses.

Determination of blood donor samples

The sIL-2R and SAA concentrations of 282 serum samples collected from apparently healthy adults are presented in Table 4.1. Overall, the median sIL-2R concentration was 448 kU/L with a 0.95 interpercentile interval of 241 – 846 kU/L. The median SAA concentration (0.95 interpercentile interval) was 2.49 (0.90 – 10.22 kU/L). For sIL-2R no gender differences were found (Mann-Whitney $U = 8665$, $p = 0.23$), whereas for SAA significant gender differences were found (Mann Whitney $U = 7285$, $p = 0.013$) as shown in Table 4.1. Furthermore, no relationship with age was found for sIL-2R ($p = 0.91$). However, the SAA showed a moderate relationship with age ($r = 0.14$, $p = 0.02$), as visible from Figure 4.2.

Analytical performance of the two evaluated assays

With respect to sIL-2R, the results were comparable to a previous study on imprecision and linearity[21]. As the assay performance was previously evaluated, we performed only a limited evaluation. Ledue *et al.* reported for sIL-2R concentrations of ca. 850 kU/L and 2000 kU/L, between run variations of respectively 3.7% and 7.0% and within run variations of respectively 3.3% and 5.1%[21]. In the present study, we additionally reported the imprecision for a pool with mean concentration of 193 kU/L. In line with the previous study[22], our analyses also revealed a low imprecision (< 4%).

To the best of our knowledge, this is the first study reporting imprecision and linearity results on the BN Prospec for SAA. As shown in the section *Results* we extensively evaluated the measuring range. Below 4.5 mg/L, the imprecision (both within- and between run) was ranging from 8.9 – 10.7%. However, the imprecision between 4.5 – 821 mg/L SAA, remained below 7.4%, suggesting a good performance of the assay. In an earlier study SAA was evaluated on the Behring nephelometer II (BNII), a previous generation of Dade Behring assays. The coefficients of variation (CV) found, for intra- and inter-assay precision were below 5.2% and 8.5%, respectively, for the three samples at concentrations representing low, normal and high[22]. Linearity for each method was within 5% of the expected values throughout the calibration range. These results are in agreement with our findings, suggesting that the performance of the SAA assay on the BN ProSpec is comparable to the same assay on the BNII. However, the agreement studies are needed to confirm the accordance between the methods, as shown in case of CRP[23,24].

Table 4.1. Evaluation of soluble interleukin-2-receptor (sIL2R) and serum amyloid A (SAA) in blood donor samples.

		sIL-2R (kU/L)[a]			SAA (mg/L)		
		All	Males	Females	All	Males	Females
Mean		468	475	456	3.22	2.96	3.60
SD		150	148	153	2.31	2.13	2.51
Minimum		189	189	208	0.77	0.77	0.78
Maximum		1058	944	1058	13.80	12.20	13.80
Percentiles	2.5	241	246	226	0.90	0.99	0.84
	25	354	368	341	1.65	1.58	2.00
	50	448	451	440	2.49	2.27	2.76
	75	557	551	563	4.03	3.51	4.84
	95	744	745	749	7.94	7.66	8.67
	97.5	846	860	812	10.22	9.87	11.4

[a] sIL-2R values were quantified in 282 (172 males and 110 females) blood donors with median age of 49 years.

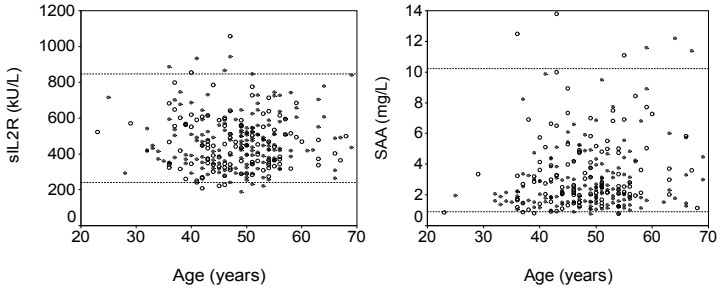

Figure 4.2. sIL-2R and SAA reference values in relation with age.
Open symbols present female values, whereas closed symbols present male values. The *horizontal lines* present reference values in the whole group.

Discussion

Reference values of sIL-2R and SAA

With respect to sIL-2R we found no sex differences and no relationship with age. The latter is in accordance with a previous study in Japanese patients[25]. As far as we know, this is the first study reporting sIL-2R values in a large population. Previous studies, often clinical trials, all used small populations (sample size < 40) to define the control group values[26-29]. Moreover, mostly no clear definition on the criteria used to select the control patients were given.

The reference values observed for SAA were comparable with the results of the previous studies[22,30]. The upper level of the reference interval (97.5th percentile) found in this study was 10.22 mg/L, whereas the median value of 2.49 mg/L is slightly higher as compared to the study of Ledue *et al.*[22]. For SAA, significant sex differences were found ($p = 0.013$), which were not reported previously. Ledue *et al.* established reference intervals on BNII in 261 adult blood donors (aged 36.2 +/– 9.0 years) with 2.5th, 50th, and 97.5th percentiles of < 0.84, 2.10 and 9.70 mg/L for SAA[22]. Yamada *et al.* reported the normal range of 0.17 – 10.0 mg/L with a self developed method based on the measurement of SAA by kinetic nephelometry[30].

In accordance with a previous study of Ledue *et al*., we observed a weak relationship of SAA with age[22]. However, in the present study this relationship appeared to be only present in males. One could argue that the sex differences observed and the sex-dependent relationship with age are simply sample related. Nevertheless, we used a large sample to establish the reference values in healthy blood-donors, in accordance to the requirements for the establishment of reference values[31,32]. The donor population was selected according to the criteria used by the Sanguine Blood Bank in Maastricht. The participants were asked to fill in a questionnaire consisting of questions considering the presence of acute or chronic illness or trauma, medication, pregnancy etc. Accordingly, donors meeting any of these criteria were excluded. The same reference population was used to establish the CRP reference values. The results were highly

consistent with a larger study using the tenfold higher sample size[33]. Therefore, we considered our sample population representative for the establishment of the reference values.

In conclusion, evaluation of the sIL-2R method on the IMMULITE and SAA method on the BN ProSpec yielded good imprecision results and satisfying linearity. The reference values established in 282 ostensibly healthy blood donors for sIL-2R were 241 – 846 kU/L. Moreover, these latter values appeared to be sex and age independent. The reference values for SAA (0.99 – 9.87 mg/L in males and 0.84 – 11.4 mg/L in females) on BN ProSpec were sex dependent and showed relationship with age.

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