High soluble interleukin-2 receptor values in Indian paediatric & adult controls – Need for population-specific threshold in the diagnosis of haemophagocytic lymphohistiocytosis

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Background & objectives: Elevated soluble interleukin-2 receptor (sIL2R) is a diagnostic criterion for haemophagocytic lymphohistiocytosis (HLH). International guidelines propose a 2400 U/ml cut-off or individual laboratory-defined cut-off. However, sIL2R normal values are so far not known in Indians. So, this study was undertaken to measure sIL2R in healthy children and adults to establish age-related reference values.

Methods: Healthy controls and cases (participants with persistent fever, organomegaly, cytopenias and biochemical markers of HLH) were prospectively enrolled. Serum sIL2R was measured by double-sandwich enzyme immunoassay in a standardization batch to determine the optimum cut-off value using receiver operator characteristic curve and was subsequently validated.

Results: One hundred and forty six age- and sex-matched children (80 controls and 66 suspected HLH cases) and 55 adults (49 controls and 6 suspected HLH cases) were prospectively enrolled. The optimal sIL2R cut-off ≥23 ng/ml was defined as raised sIL2R in the standardization batch. No controls had sIL2R ≥23 ng/ml in the validation batch. In healthy controls, median sIL2R (interquartile range) decreased with increasing age from 9.0 ng/ml (6.6-13.4) below five years of age to 3.2 ng/ml (2.8-5.1) in adults. Proposed upper limit of normal value for sIL2R is 17.4 ng/ml in less than five year, 12.2 ng/ml in 5-9 yr, 6.7 ng/ml in 10-17 yr and 5.2 ng/ml in ≥18 yr. sIL2R accuracy to diagnose HLH marginally improved with age-appropriate cut-off.

Interpretation & conclusions: Paediatric controls in India showed higher sIL2R levels than most studies conducted in other countries, except for some reports in Chinese and Russian populations. Age-appropriate reference values of sIL2R in a specific population may be considered to determine elevated sIL2R as a marker of HLH.

Key words Enzyme-linked immunosorbent assay - haemophagocytic lymphohistiocytosis - India - reference values - soluble CD25 - soluble interleukin-2 receptor

One of the increasingly frequent causes of fever, organomegaly and cytopenia in paediatric and adult patients is haemophagocytic lymphohistiocytosis (HLH)¹. HLH is a cellular immune dysregulation

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resulting from underlying genetic defects or triggered by severe infection [infection-associated haemophagocytic syndrome (IAHS)], malignancy [malignancy-associated haemophagocytic syndrome (MAHS)] or rheumatological condition (macrophage activation syndrome)\(^1\).

HLH 2004 diagnostic criteria\(^2\) are either the presence of a familial disease or a known genetic defect or the presence of five out of eight diagnostic criteria, which include fever, splenomegaly, bicytopenia, hyperferritinaemia, hypertriglyceridaemia and/or hypofibrinogenaemia, haemophagocytosis in bone marrow or spleen or lymph nodes, decreased or absent NK cell activity and raised soluble CD25 [soluble interleukin-2 receptor (sIL2R)]. Elevated levels of sIL2R were first documented in HLH in 1989\(^3\) and are widely found in HLH. Induced by uncontrolled and ineffective immune activation by lymphocytes, sIL2R is a laboratory indicator of in vivo immune system activation. The suggested threshold for raised sIL2R is either 2400 U/ml or above normal limits as per laboratory-specific threshold\(^2,4\). There is no clearly defined relation between ng/ml and U/ml concentrations, and both units have been used in published reports, but mostly U/ml\(^5\). sIL2R levels in healthy individuals are known to be higher in children than in adults and decrease with increasing age\(^5,6\). However, this assay is not commonly available in India. To the best of our knowledge, age-wise sIL2R serum concentrations in healthy Indian subjects are not known, so the dilemma is that, to which levels the diseased subjects should be compared. Thus, a prospective study was conducted to establish the normal range of sIL2R in the serum of North Indian children and young adults and the age-specific cut-off value for elevated sIL2R as a marker of HLH.

**Material & Methods**

A descriptive/cross-sectional study was conducted at Sir Ganga Ram Hospital, a tertiary care hospital in New Delhi, from January 2014 to March 2018 after obtaining approval from the Institutional Ethics Committee.

**Selection of study participants:** Children and adults with persistent fever, organomegaly, bicytopenias and biochemical markers of HLH were labelled as HLH cases as per HLH 2004 criteria\(^2\) and sampled prior to initiation of immunosuppressive therapy. Healthy age-matched controls included children undergoing elective surgery (circumcision and correction of congenital anomalies) or HLA typing and children consulting for minor non-febrile illness (iron deficiency anaemia, immune thrombocytopenia and acute gastritis) as well as adult blood donors.

**Sample collection and processing:** After obtaining written consent from adults/guardians or child’s assent as applicable, blood samples collected in plain vials were centrifuged and serum was stored at −20°C until testing. Both plasma and serum samples were compared while standardizing the assay. Serum samples showed lower within-assay variation, hence, serum was the sample type chosen for the study. A double-sandwich enzyme-linked immunosorbent assay (Human sIL-2R Platinum ELISA, eBioscience, San Diego, CA, USA) was used to measure sIL2R, running all standards (serial dilutions from 20 to 0.625 ng/mL) and test samples in duplicates. Optical density was analyzed with Infinite 200 PRO ELISA plate reader (Tecan, Switzerland). As no predefined equivalence between nanogram and international units was given in the kit, and due to varied conversion methods found in the literature, receiver operating characteristic (ROC) curves were used in a first batch of cases and controls (standardization batch) to determine the optimal cut-off value of sIL2R distinguishing cases from controls (area under the curve: 0.954). This cut-off was further tested in a validation group of cases and controls. Upper limit of normal (ULN) value was calculated as median+2 standard deviations (SDs).

**Statistical analysis:** Statistical analysis was conducted using SPSS Statistics for Windows, Version 17.0 (SPSS Inc. IBM Corp. Chicago, IL, USA). Age-wise distribution of sIL2R in healthy controls was assessed. Pearson’s Chi-square or Fisher’s exact test was used to compare categorical variables. Normal range in each age group was defined as mean±2 SDs. ULN value was defined as median sIL2R + 2 SDs in each age group. Mann–Whitney U test was used to compare sIL2R levels in various groups, after confirming non-normality using Shapiro–Wilk test. Continuous variables were compared using Spearman’s rank correlation.

**Results**

A total of 207 individuals were prospectively enrolled: 146 children (80 controls and 66 cases)
and 55 adults (49 controls and 6 cases). Cases and controls had similar age/sex distribution (Table I). The standardization batch included 12 cases and 18 controls. ROC curve identified the optimal sIL2R cut-off to segregate cases from controls as 23 ng/ml. This cut-off in the validation batch showed sIL2R ≥ 23 ng/ml in 41 (68.3%) out of 60 cases of suspected HLH and none of the 111 controls (P < 0.001). Between-assay and within-assay variation coefficients were 12.4 and 6.3 per cent, respectively.

**Soluble interleukin-2 receptor in healthy controls:** In healthy controls, sIL2R showed a significant inverse relationship with age (r = −0.7, P < 0.001; Table II), as reported previously. Median sIL2R levels were significantly higher in paediatric controls than in adult controls (Table I). Our paediatric control selection included 39 healthy children and 41 children with non-febrile minor illnesses. Both control groups were compared in every age group, and sIL2R levels showed no significant difference (Supplementary Table). ULN values are given in Table II.

**Soluble interleukin-2 receptor in cases with haemophagocytic lymphohistiocytosis:** Children and adults with suspected/confirmed HLH included 18 cases of primary HLH (3 with PRF-1 deficiency and 15 with positive family history), 27 cases of IAHS (EBV – 7, dengue – 5, enteric fever – 3, tuberculosis – 2 and 10 cases of other viruses or bacteria), 10 cases of macrophage activation syndrome (6 – juvenile idiopathic arthritis, 3 – systemic lupus erythematosus and 1 – Kikuchi’s disease), 5 cases of MAHS (4 – lymphomas and 1 – Wilms’ tumour) and 12 cases of unclassified HLH. Clinical symptoms and laboratory findings in patients with HLH are shown in Table III.
As expected, HLH cases had significantly higher sIL2R levels than controls in all age groups (Table IV). The proportion of cases with sIL2R ≥23 ng/ml decreased from 84.4 per cent in children below five years to 50 per cent in adults. When the ULN was used to define elevated sIL2R for age, 86.1 per cent of HLH cases had raised sIL2R.

**Table IV. Serum soluble interleukin-2 receptor in patients with haemophagocytic lymphohistiocytosis (n=72)**

| Age group (yr) | n  | Median (ng/ml) | IQR | Range | sIL2R >ULN, n (%) |
|---------------|----|----------------|-----|-------|------------------|
| <5            | 32 | 38.8**         | 29.2-52.6 | 9.5-130 | 28 (87.5) |
| 5-9           | 14 | 29.9**         | 14.8-47.8 | 5.0-83.5 | 11 (78.6) |
| 10-17         | 20 | 27.7**         | 10.8-51.1 | 4.7-80.9 | 18 (90.0) |
| ≥18           | 6  | 25.7*          | 8.5-30.8  | 2.6-43.4 | 5 (90.0)  |
| Total         | 72 | 33.9**         | 20.6-49.9 | 2.6-130  | 62 (86.1) |

*P <0.01, **<0.001, Mann–Whitney U test comparing patients with HLH and controls. All values are in ng/ml

Discussion

At present, no reference values of sIL2R are available from the Indian population. Although there are a few studies on sIL2R in various disease conditions including healthy controls, sIL2R levels vary greatly among the published reports, many of which measure it in U/ml, with varied conversion factors to ng/ml. Decreasing sIL2R levels with increasing age in healthy individuals has been previously reported.5,6

The normal range of sIL2R in healthy adults in our study agrees with a previous report from Russia, in which 16 adult controls had a median (range) serum sIL2R of 3.3 ng/ml6 (Table V). However, Japanese controls had a lower serum sIL2R in children and in adults with ages similar to our series.7 In China, Gao et al8 too reported low levels of sIL2R in children aged 2-3 yr. Two reports used the same manufacturer as the present study, Zhao et al9 described serum sIL2R in Chinese children aged 3-12 yr similar to the present study, while Deveci et al10 reported considerably lower sIL2R than the present study9,10 (Table V). Thus, both children and adults from India seem to have higher serum sIL2R concentrations than most reports.

As a proportion of our paediatric cases, the study controls were not strictly healthy and may have had some degree of inflammation, this could have caused higher sIL2R levels; however, the subgroup of controls with minor non-febrile illness and elective surgery had the same levels as children sampled for HLA typing. This is a major limitation of this study.

Another possible reason for higher sIL2R levels in Indian controls may be a high parasitic load, since children living in tropical countries are more prone to gastrointestinal infections and parasitic disease11,12.
Recently, elevated sIL2R levels was found to correlate with severity of malaria. Data from this study will help in interpretation of sIL2R testing in suspected cases with HLH in India. Further studies may demonstrate whether specific cut-offs would help identify HLH in patients with fever, infection or autoimmune disease.

Overall, our study confirms that elevated sIL2R levels is a good marker of HLH. sIL2R levels in healthy subjects are higher in younger children and decrease with increasing age. Normal values should be assessed by the method intended to be used in patients. Determining age-appropriate sIL2R cut-offs is absolutely essential to define increased sIL2R in a specific population.

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**Conflicts of Interest:** None.

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**Table V. Studies reporting soluble interleukin-2 receptor quantitation in healthy controls**

| Studies                | Country | n   | Age (yr)         | Sample | sIL2R          |
|------------------------|---------|-----|------------------|--------|----------------|
| Paediatric controls    |         |     |                  |        |                |
| Gotoh *et al*, 1999    | Japan   | 56  | 5.3±3.8* (1-14)  | Serum  | 1.5±0.9 ng/mla |
| Gao *et al*, 2015      | China   | 14  | 1.6 (1-3.3)b     | Plasma | 0.99 (0.8-1.1) ng/mlb |
| Deveci *et al*, 2014   | Turkey  | 38  | 10.2±3.7a       | Serum  | 0.6 (0.1-2.6) ng/mlb |
| Zhao *et al*, 2018     | China   | 64  | 6.7±1.4* (6-12)  | Serum  | 7.3±2.2 ng/mlb,c |
| Present study          | India   | 86  | 6.4±4.6* (0.2-17)| Serum  | 6.7 (4.6-9.4) ng/mlb |
|                       |         |     |                  |        | 7.7±4.1 ng/mlb,d |
| Adult controls         |         |     |                  |        |                |
| Gotoh *et al*, 1999    | Japan   | 38  | 37±11* (22-67)   | Serum  | 0.7±0.3 ng/mlb |
| Barabanshikova *et al*, 2017 | Russia | 16  | 56 (50-67)b     | Serum  | 3.3 (0.9-5.8) ng/mlb |
| Present study          | India   | 49  | 34.4±8.8* (20-52)| Serum  | 3.2 (2.8-5.1) ng/mlb |
|                       |         |     |                  |        | 3.8±1.4 ng/mlb,d |

*aMean±SD; bMedian (range); cThe authors wrongly reported pg/ml, but the kit mentioned measures sIL2R in ng/ml (assay range 0.31-20 ng/mL); dMean±SD is given for comparison with other studies, but data are non-normally distributed.*
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