ORIGINAL ARTICLE

Clinical relevance of soluble HLA class I molecules in Waldenstrom Macroglobulinemia

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Abstract

Objectives: Waldenstrom Macroglobulinemia (WM) is a B-cell neoplasm characterised by secretion of IgM by lymphoplasmacytic bone marrow cells and by cytopenias and hypogammaglobulinemia in a subset of patients. Beta-2 microglobulin (b2m) is a major prognostic factor in WM and the heavy chain of HLA class I molecules, which are known to have immunosuppressive properties and have been implicated in the pathogeny of several malignancies. *Methods:* We assessed the serum levels of the total soluble HLA-I molecules and the HLA-Gs molecules in 105 patients with IgM-related disorders [WM ($n = 42$) and IgM MGUS ($n = 63$)], and compared the results to 41 healthy subjects. *Results:* We found higher levels of HLA-Is in WM, compared to IgM MGUS and healthy donors. HLA-Gs levels were similar in WM and in IgM MGUS, but higher than in healthy donors. The association between HLA-Is at the cut-off of 1.8 µg/mL and known markers of poor prognosis was then evaluated among WM patients using univariate and multivariate methods. Based on this, high HLA-Is level was strongly associated with high serum β2M level >3 mg/L [OR = 2, (CI 95% 1.1–5.7); $P = 0.04$], age > 65 yrs [OR = 1.5, (CI 95% 0.5–4.1), $P = 0.06$] and haemoglobin ≤11.5 g/dL [OR = 3.3, (CI 95% 1.2–9.7); $P = 0.03$]. High levels of serum HLA-Is were also found in patients with cryoglobulinemia, however irrespectively of WM or IgM-MGUS status. *Conclusion:* Together our results suggest a possible role for soluble MHC class I molecules in WM disease. Further investigations are necessary to further demonstrate the prognostic impact of soluble MHC class I molecules in Waldenstrom Macroglobulinemia.

Key words Waldenstrom Macroglobulinemia; IgM-monoclonal gammopathy of undetermined significance; serum total soluble HLA-I molecule; serum HLA-Gs molecule

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Waldenstrom's Macroglobulinemia (WM) is a B-cell neoplasm characterized by excessive secretion of IgM by lymphoplasmacytic bone marrow (BM) cells (1). The accurate diagnosis of WM is sometime difficult because of the overlapping heterogeneity of presentation at diagnosis between IgM-monoclonal gammopathy of undetermined significance (MGUS), smoldering or asymptomatic WM and

symptomatic WM (2–4). Few markers are reliable in routine to monitor the tumor burden in WM, and the common ones remain the serum IgM paraprotein level, the bone marrow involvement by clonal lymphoplasmacytic cells, the serum viscosity and the presence of cytopenias.

Another characteristic of WM disease is the presence of hypogammaglobulinemia in the patients at diagnosis

(5), independently of the presence of cytopenia. Both hypogammaglobulinemia and cytopenias are thought to be multifactorial, partly due to clonal expansion in the BM, although infiltration of organs such as liver and spleen can be incriminated in some patients (6, 7). Recently, Hunter *et al.* eventually ruled out BM infiltration in a large series of 197 patients studied to determine the exact incidence of hypogammaglobulinemia. They also reported that response to therapy didn't restore hypogammaglobulinemia in WM patients. They proposed a possible constitutive Ig dysregulation in WM (8).

Non-classical HLA-I molecules are predominantly expressed as cell surface proteins of almost all nucleated cells, but existence of non-cell-bound soluble HLA molecules, soluble HLA-I (sHLA-I), has also been described (9). Similarly to their membrane bound counterpart, sHLA-I molecules is composed of a heavy chain non-covalently bound to a beta2-microglobulin light chain. The main soluble HLA class I molecules (HLA-Is) is HLA-G5 (also called HLA-Gs) (9). HLA-Is as well as HLA-Gs are known to have immunosuppressive properties (10) and have been implicated in the pathogeny of several malignancies (9), including in some lymphoproliferative disorders (11–14). An immunomodulating role partially explained by their capacity to protect target cells from NK and T cytolysis and to trigger apoptosis in activated T CD8$\pm$ cells (15, 16). Furthermore, the serum b2m is the light chain of the HLA class I molecular complex, expressed on the surface of the majority of nucleated cells (17) and corresponds to the serological soluble HLA heavy chain-free b2m (18). Since serum b2m is an important feature in MM, other HLA class I molecules may be of interest in monoclonal gammopathies.

In B-CLL, HLA-G expression has been correlated to the degree of immunosuppression and to prognosis but authors did not study HLA-Is (13). On the other hand, while HLA-Is appeared to be a tumor burden marker and a powerful prognostic marker, especially in combination with b2m in Multiple Myeloma, HLA-Gs had no survival prognostic impact, but was a marker of clonality since high levels of HLA-Gs were observed in both IgG and IgA MGUS and MM patients (19). It is therefore of importance to study the expression and potential role of these molecules in WM disease, known to be at the crossroad between low grade non Hodgkin B cell lymphoma and MultipleMyeloma.

We hypothesized therefore, that expression of soluble HLA class I molecules, through their strong immunosuppressive properties, may be involved in the pathogeny of WM disease, and help to further explain the cytopenias and hypogammaglobulinemia phenomena.

Patients and methods

Patient selection

A total of 105 patients with WM ($n = 42$) or IgM-MGUS ($n = 63$) were studied. The diagnosis of WM was according to the recommendations of the Second International Workshop on Waldenstrom's Macroglobulinemia (1). Patients who had an IgM-monoclonal protein of less than 10 g/L and less than 5% involvement in the BM with lymphoplasmacytic cells were diagnosed as IgM-MGUS (20). Approval for the review of these records was obtained from the CHRU Lille Institutional Review Boards and was in accordance with the Declaration of Helsinki. Finally, 41 age-matched healthy subjects were also studied.

Laboratory parameters

The biological evaluations were performed in Lille for usual parameters and Rennes for soluble HLA-I class molecules. HLA-Gs and HLA-Is concentrations were measured as previously described (19). Briefly, specific sandwich ELISA. Microtiter plates (Corning Costar, France) were coated in phosphate-buffered saline (PBS) at pH 7.4 with MEM-G/9 (10 µg/mL, Exbio, Czech republic) with a monoclonal antibody (MoAb) detecting specifically HLA-Gs molecules associated with b2m (Exbio, Czech rep.) or with W6/32 (8 µg/mL) MoAb recognising a monomorphic determinant any of the HLA class I heavy chains associated to b2m (Harlan Sera-Lab Limited, England) (21). After washes and saturation, pure sera for HLA-Gs or diluted at 1/50 for HLA-Is were added to each well and tested in triplicate. After incubation and washes, anti-b2m-horse-radish-peroxidase (Dako, France) was added to each well and plates were incubated. Plates were washed then incubated with the substrate (OPD, orthophenylenediamine dihydrochloride, Dako, France). After addition of H_2SO_4 (1N), optical densities were measured at 490 nm. Standard curves were performed as described (19). Soluble HLA molecules (Sangstat) were utilized as a standard to calculate the total amount of sHLA-I antigens. Thus, the concentrations of HLA-Gs and HLA-Is were determined from the value of optical density according to the standard curves.

Statistical analysis

To describe the distribution of HLA-Is and HLA-Gs levels, the median and interquartile range (IQ range) are reported. Median values are compared using Wilcoxon rank-sum test. To establish a cut-off value for serum HLA-Is that would differentiate the WM and the IgM-MGUS patients, we applied to our series different HLA-Is thresholds, either previously defined in MM

2.1 μg/mL (19), or corresponding to the upper 90th percentile of the IgM-MGUS group (1.8 μg/mL). Using this cut-off, the HLA-Is for WM was dichotomized as low (≤1.8 μg/mL) vs. high (>1.8 μg/mL). Fishers's exact test was used to compare proportions. To adjust for multiple variables in a model, a logistic regression model was used to evaluate the association between dichotomized HLA-Is and known tumor burden measures or prognostic factors. The following variables were evaluated: age, hepatosplenomegaly (assessed either clinically or by imaging studies), hemoglobin (Hb), leukocyte cell count (WBC), neutrophils and lymphocytes count, platelets count, monoclonal IgM level, and serum β2-microglobulin (β2M). We also studied serum IgA and IgG levels. The odds ratio (OR) and the 95% confidence intervals (CI 95%) are reported. All statistical tests are two-sided. All analyses were performed with SPSS software.

Results

Patient characteristics

Patient characteristics are described in Table 1 for the 105 patients. Of these, 42 had WM and 63 had IgM-MGUS. Median age was 64.5 and 62 yrs for patients with WM and IgM-MGUS, respectively. Median BM Involvement (%) was 30 (17.7–53.7). In the patients with WM, 19 received prior therapy with a median duration from last therapy to serum collection of 1 yr (range, 6–30 months). All serum samples were collected and stored prior to start therapy. The treated patients received

Table 1 Patient characteristics

Characteristic	WM, *n* = 42	IgM-MGUS, *n* = 63
Age (yrs)		
>65 [*n* (%)]	19 (45)	25 (40)
Median (range)	64.5 (37–90)	62 (39–85)
	n (%)	
Males	27 (64)	41 (65)
Kappa light chain	31 (74)	46 (73)
Hepatosplenomegaly	3 (7)	–
IgM related disorders		
Cryoglobulinemia	15 (35.7)	4 (6.3)
Hemoglobin (g/dL)*		
<10	8 (19)	2 (3.2)
≤11.5	15 (36)	5 (8)
WBC (<4 × 10⁹/L)	5 (12)	3 (5)
Platelets (<100 × 10⁹/L)	6 (14)	2 (3.2)
IgM (g/L)		
>40	10 (23)	–
ß2m (>3 mg/L)*	13 (32.5)	6 (10.7)

Degree of significance, *P < 0.001. HLA-Is, total soluble HLA class I. HLA-Gs, soluble HLA-G. IgM normal range is 0.3–2.1 g/L. b2, beta-2.

either alkylating agents (*n* = 15) or fludarabine (*n* = 4) with or without rituximab. At the date of point, six patients (9%) with IgM MGUS had progressed to malignancies.

Higher serum levels of soluble HLA molecules in IgM-related disorders than in the control group

HLA-Is (μg/mL) concentrations varied between IgM disorders and the control group (Fig. 1A). A significant difference was observed between patients with WM and IgM MGUS or the control group, medians (IQ range) were 1.75 (1.1–2.1) vs. 0.89 (0.5–1.2) and 0.69 (0.4–0.8) μg/mL, respectively (*P* < 0.001). Serum HLA-Is levels were not significantly different between controls and IgM MGUS. Interestingly, the median of serum HLA-Is levels (μg/mL) was 1147.3 and 814.0 in IgM MGUS patients
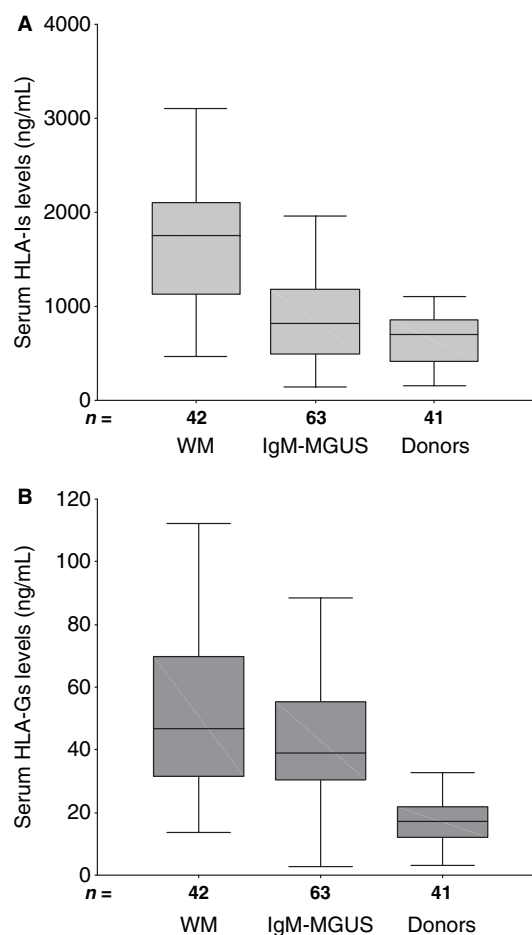

Figure 1 Repartition of soluble HLA class I molecules between Waldenstrom's Macroglobulinemia (WM) and IgM-MGUS. (A) Repartition of serum HLA-Is (ng/mL) levels between patients with Waldenstrom's Macroglobulinemia (WM), IgM-MGUS and healthy donors (donors) (*P* < 0.0001). (B) Repartition of serum HLA-Gs (ng/mL) levels between patients with Waldenstrom's Macroglobulinemia (WM), IgM-MGUS and healthy donors (donors) (*P* < 0.0001).

that evolved towards malignancy ($n = 6$) vs. those that did not ($P \leq 0.01$). Regarding serum HLA-Gs levels (ng/mL) (Fig. 1B), there was no variation between IgM-MGUS and WM patients, 40.9 (30–66) and 46 (29–71) ng/mL, respectively. The only difference was evidenced between controls [median HLA-Gs serum levels of 17.3 (12–21)] and IgM-related disorders ($P < 0.001$). No correlation was observed between HLA-Is and HLA-Gs and both HLA markers did not correlate to creatinine.

Serum HLA-Is level correlates with markers of adverse prognosis in WM

The distinction of patients with active WM disease that require therapy from those who do not require therapy is often challenging given that some patients may have significant symptoms with minimal IgM levels and vice versa. We categorized the variables studied in this series according to cut-offs recently proposed in the International Prognostic Scoring System for WM (IPSS) age (>65 yrs), β2M (>3 mg/L), hemoglobin (≤11.5 g/dL), platelet (<100 × 10^9/L) and serum IgM (<70 g/L) (22), and previously published markers including hemoglobin (<10 g/dL), WBC (<4 × 10^9/L) and serum IgM (<40 g/L) to separate patients with markers of high tumor burden and poor prognosis (6, 23). In our series, 19 (45%), 15 (36%) and eight (19%) patients with WM had low, intermediate and high risk in the WM-IPSS scoring system, respectively. The median (μg/mL, IQ range) serum HLA-Is level were 2.07 (1.7–2.2), 1.60 (1.2–2.0) and 1.30 (0.9–1.7), respectively ($P = 0.05$). As shown in Table 2, median HLA-Is levels were higher

Table 2 HLA-Is levels (μg/mL) by categorized variables for patients with Waldenstrom Macroglobulinemia

	Median (IQ range)	P
b2m (>3 mg/L)		0.05
No	1.6 (0.9–1.9)	
Yes	2 (1.5–2.3)	
Age (>65 yrs)		0.04
No	1.5 (0.9–1.8)	
Yes	2.1 (1.3–2.3)	
WBC count (<4 × 10^9/L)		NS
No	1.5 (1.1–1.9)	
Yes	1.7 (1.1–2.1)	
IgM (>40 g/L)		NS
No	1.6 (1.3–1.9)	
Yes	1.8 (1.1–2.1)	
Hemoglobin (<10 g/dL)		NS
No	1.5 (0.9–1.8)	
Yes	1.8 (1.1–2.1)	
Platelets count (<100 × 10^9/L)		NS
No	1.6 (1.1–1.9)	
Yes	1.8 (1.1–2.1)	

IQ range, interquartile 25–75; b2, beta-2; WBC, white blood count.

($P \leq 0.05$) in patients with adverse prognosis markers (age > 65 yrs and β2M > 3 mg/L), but not higher tumor burden (serum IgM level > 40 g/L, and hemoglobin < 10 g/dL or ≤11.5 g/dL) in patients with WM. Median serum HLA-Is level was marginally higher for the WM patients with hepatosplenomegaly, with low neutrophils and lymphocyte counts and low serum IgA levels.

HLA-Is level higher than 1.8 μg/mL is associated with markers of adverse prognosis

In order to use the HLA-Is level measurement in clinic and to identify patients with WM, we sought to determine a cut-off value that would identify patients that display poor prognosis markers. The upper 90th percentile of serum HLA-Is level among the IgM-MGUS group (1.8 μg/mL) was selected as the cut-off, and applied to the WM group. The group of patients with higher serum HLA-Is level, determined as serum HLA-Is > 1.8 μg/mL ($n = 19$), had significantly higher percentage of patients with elevated levels of serum β2m (41.5% vs. 20%, $P = 0.035$) and an age > 65 yrs (60% vs. 29.5%, $P = 0.03$). The serum HLA-Is level was elevated for all three patients with clinical hepatosplenomegaly.

In the logistic regression model, factors evaluated for association with high serum HLA-Is level were age (>65 yrs), hemoglobin (<10 g/dL or ≤11.5 g/dL), platelet count (<100 × 10^9/L), white blood cell count (<4 × 10^9/L), β2M (>3 mg/L), M-protein (>40 g/L). Modeling results showed a significant association of serum HLA-Is level with elevated β2M, age and hemoglobin, with 21% (6/29) vs. 46% (6/13) of patients with high serum HLA-Is level in β2M ≤3 mg/L vs. >3 mg/L [OR = 2, (CI 95% 1.1–5.7); $P = 0.04$], with 30% (7/23) vs. 53% (10/19) of patients with high serum HLA-Is level in age ≤65 yrs vs. >65 yrs [OR = 1.5, (CI 95% 0.5–4.1), $P = 0.06$] and with 22% (6/27) vs. 53% (8/15) of patients with high serum HLA-Is level in hemoglobin > 11.5 g/dL vs. ≤11.5 g/dL [OR = 3.3, (CI 95% 1.2–9.7); $P = 0.03$].

WM patients with cryoglobulinemia syndrome have higher serum HLA-Is level

Waldenstrom Macroglobulinemia disease is characterized by wide clinico-biological heterogeneity of symptoms, including complications such as various autoimmune diseases, related or not to the IgM serum monoclonal protein. Auto-immune hemolytic anemia (AHAI) and neuropathy are the most frequent (2, 4). We recorded the presence of autoimmune IgM related disorders ($n = 28$) in the WM and IgM MGUS populations. We observed eight sensory-motor neuropathies with MAG antibodies,
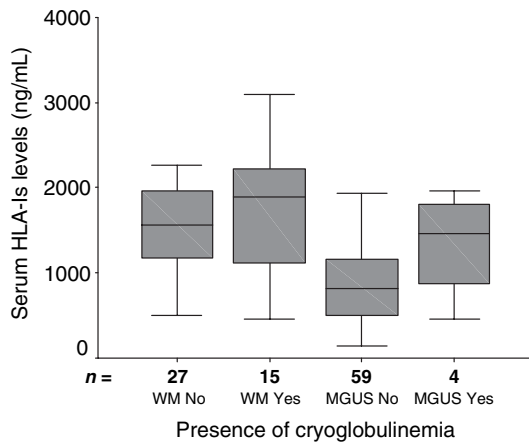
Figure 2 Serum soluble HLA-Is levels in patients with and without cryoglobulinemia in the WM population and in the IgM-MGUS population ($P = 0.014$).

21 cryoglobulinemia syndromes, and two autoimmune haemolytic anemias. No patient presented with autoimmune thrombocytopenic purpura and one patient with glomerulonephritis has been removed, as there was no evidence for a role of IgM, although highly suspected. It is important to determine markers able to isolate those subsets of patients with WM that develop these complications.

We noticed higher levels of serum HLA-Is, but not HLA-Gs, in WM patients that presented with cryoglobulinemia ($P = 0.014$). Interestingly, this was also observed in IgM-MGUS patients (Fig. 2). The median (IQ range) of serum HLA-Is levels (μg/mL) was 2.5 (1.5–2.7) and 1.57 (1.1–2.0) in patients with WM, and 1.47 (0.6–1.8) and 0.80 (0.5–1.1) in patients with IgM MGUS, with and without cryoglobulinemia, respectively in each group. No relationship was found with any other autoimmune disease, as well as with occurrence of IgM amyloidosis, a complication of poor prognosis in WM disease.

Discussion

The role of non-classical soluble MHC class I molecule in lymphoproliferative disorders has already been reported (13, 14, 19). Soluble HLA class I molecules have been implicated in the pathogeny of several malignancies through their immunosuppressive properties (9). Our study suggests that HLA-Is is a new marker of WM tumour load, with higher levels found in patients with WM compared to IgM-MGUS and healthy controls. Furthermore, serum HLA-Is levels correlated to markers of poor prognosis in WM disease, especially b2m and age. These results have to be confirmed in a larger series. The soluble HLA class I complex, a couple of b2m and

the HLA class I heavy chain, has been studied as serological b2m-free HLA class I heavy chain (FHC) (24) and as a total soluble HLA class I complex, the latter being more stable (19, 25). Similar results were found in Multiple Myeloma (MM), another malignant plasma cell disorder (19). These results underline the close dysfunction of the immune system, in some extent, in the pathogenesis of WM and MM diseases. Future studies will have to consider the ratio of the serum HLA-I levels over the cell surface expression, and not only the soluble form, since the prognostic impact of the ratio appears to be higher than either parameter alone (26).

Inversely, HLA-Gs was higher in all kinds of monoclonal gammopathies compared to controls. Several studies have confirmed the increased expression of HLA-G either on the cell surface or in the serum in B cell disorders (14, 27). This is concordant with our results in both MM and WM diseases. Interestingly, the serum HLA-Gs levels were close in MM and WM. Those results favour expression of HLA-Gs has an early event in clonal development, and is concordant with the known immunosuppressive effect of HLA class I molecules.

In our study, survival was not an endpoint related to the short follow-up and the prolonged survival of WM patients. Larger study is then necessary to answer this question, however, one must consider studying HLA-Is, but not HLA-Gs. Our previous study with MM already showed that HLA-Gs had no impact on survival prognosis or relation to tumour burden markers (19, 28). Interestingly, the survival impact of HLA-Gs had only been demonstrated in B cell malignancies when the membranous form was studied (13). That impact is apparently lost when the soluble form is concerned (13). It would be also very interesting in the future to determine whether soluble HLA molecules, especially HLA-Is, is a marker of progression from MGUS to either WM or MM diseases. IgM-MGUS displays at least 2-fold increase risk of progressing towards neoplasy, especially WM disease, compared to IgG and IgA-MGUS (29, 30). Study of soluble HLA molecules in MGUS may help better understand this discrepancy between Ig isotypes MGUS regarding the risk to evolved towards malignancies.

High incidence rate of infection has also been related to higher expression of HLA soluble molecules through their immunosuppressive properties (13). Although it was not possible to study the occurrence of infectious episodes, as our population was mostly asymptomatic and untreated, we studied the relationship of soluble HLA class I molecules with cytopenias and hypogammaglobulinemia (data not shown). We found that HLA-Is and HLA-Gs did not correlate to cytopenia and hypogammaglobulinemia. The immunosuppressive properties of soluble HLA molecules were partially explained by their

capacity to protect target cells from NK and T cytolysis but also to trigger apoptosis in activated T CD8+ cells (31). If the relationship between soluble HLA molecules and markers of cellular immunity was strong, it seems that soluble HLA molecules do not affect humoral immune deficiency.

Autoimmune complications, such as cryoglobulinemia, are not infrequent in IgM-related disorders (32). In our study, occurrence of cryoglobulinemia positively correlated to HLA-Is, although the incidence of cryoglobulinemia is lower in the IgM MGUS population in our series that might have introduce a bias in the analysis. Interestingly this relationship was observed in both patients with WM and with IgM MGUS (i.e. irrespective of tumor burden), which is not surprising knowing that the occurrence of those complications is not tumor burden dependent but linked to the presence of the IgM component and that the incidence of this complication is quite similar in IgM MGUS and WM (33). An increase serum HLA-Is level was reported in patients with auto-immune diseases (9), including cryoglobulinemia, although in the particular context of hepatitis C (34). A deregulated Th1/Th2 balance, in favor of Th1, leading to toxic effects induced by IFNγ, that increases production of soluble HLA molecules, has been proposed (34, 35). Although there is no report of study of T cell compartments in WM, increased percentage of Th1 cytokine-secreting T cells was found in MGUS and MM (36). It would be then of interest to determine production of Th1 and Th2 cytokines in IgM disorders and study the relationship with soluble HLA class I molecules.

In summary, we demonstrate that serum HLA-Is level, a new immunosuppressive molecule, differentiates patients with MGUS from those with WM. It is related to poor prognostic markers that isolate WM patients with progressing disease who require therapy. Future studies are needed to determine the value of serum HLA-Is level on survival as a prognosis marker. These findings have therapeutic implications in the management of patients with WM.

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Author contribution

XL, IMG, SPT, TF, EZ: Designed the project, Review manuscript. ASM, XL, VC, TG: Studied the patient charts. AD: Performed statistics. YS, GLF, LA, AMR, NB, MF, AWH, SP, ZRH: Performed research. BH: In charge of collection and storage of samples.

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