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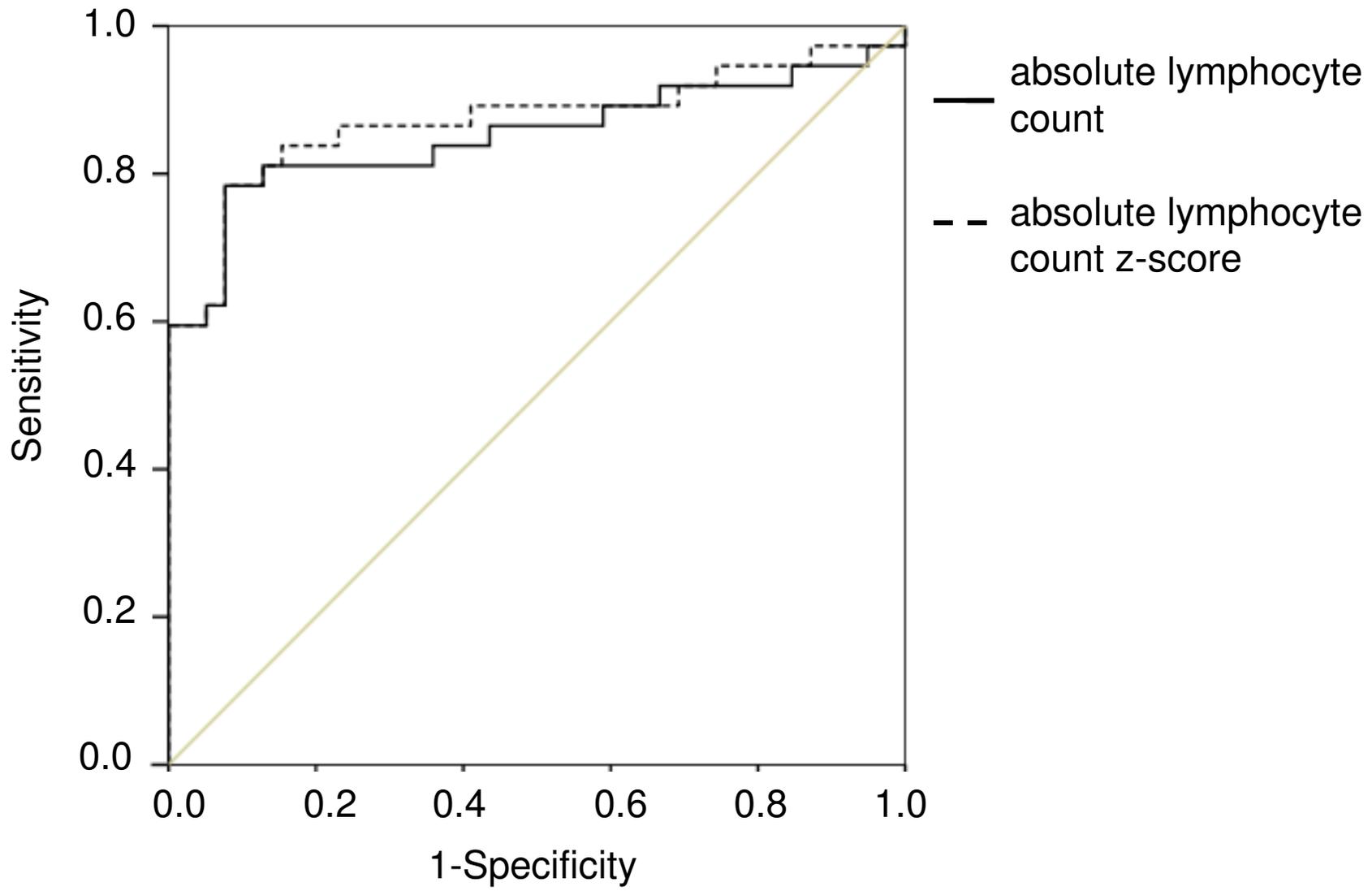


## Lymphocytopenia as a marker for pandemic influenza A/H1N1 2009 virus infection in children

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3 **Lymphocytopenia as a marker for pandemic influenza A/H1N1 2009 virus infection in**  
4 **children**  
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60**Abstract**

Lymphocytopenia has been reported in adults with pandemic influenza A/H1N1 2009 infection, but data in children are inconclusive. Data from 76 children presented with flu-like symptoms between July and November 2009 and tested for pandemic influenza A/H1N1 2009 virus and white blood cell (WBC) counts were analyzed. Samples from thirty-seven (48.7%) children resulted in a positive PCR assay for pandemic influenza A/H1N1 2009 virus. When comparing data from these children with data from 39 (51.3%) children with uncomplicated flu-like illness and negative PCR assay for pandemic influenza A/H1N1 2009 virus, no difference in disease duration, median age, red blood cell count, haemoglobin concentration, C reactive protein concentration, and absolute neutrophil count was observed, whereas significant differences were apparent when considering WBC count, relative and absolute lymphocyte count, absolute lymphocyte count z-score, and platelet count. Receiver operating characteristic curve analysis revealed that the best absolute lymphocyte count and absolute lymphocyte count z-score cut-points that simultaneously maximized sensitivity and specificity were 2,256 cells/ $\mu$ L and -0.89, respectively, sensitivity being 0.81 (95%CI:0.68-0.94), specificity 0.87 (95%CI:0.77-0.98), positive predictive value 0.85 (95%CI:0.74-0.97), and negative predictive value 0.83 (95%CI:0.71-0.94).

In conclusion, lymphocytopenia is a marker for influenza A/H1N1 2009 virus infection in children. Absolute lymphocyte count  $<2556$  cells/ $\mu$ L or absolute lymphocyte count z-score  $<-0.89$  may be useful cut-offs to discriminate against children at higher risk of infection during epidemics. Considering that the pandemic virus is highly likely to continue to circulate in the coming winter season, these findings provide direct and practical implications for the near future.

## Introduction

Lymphocytopenia has been reported to be a marker for pandemic influenza A/H1N1 2009 virus infection in adults (Cunha et al. 2009). This is consistent with previous studies reporting the occurrence of lymphocytopenia in adults and children with other types of influenza A virus infections (Liem et al., 2009; Lupovitch A., 2005). Influenza-mediated lymphocytopenia has been also investigated as a possible cause of increased susceptibility to bacterial super-infection (Stegemann et al., 2009). Data from children with flu-like illness that had been referred to a paediatric Infectious Disease Unit between July and November 2009 were analyzed; nasopharyngeal swabs samples were taken and tested for pandemic influenza A/H1N1 2009 virus by polymerase chain reaction PCR), and white blood cell (WBC) counts were also measured. The data collected allowed the for the evaluation of the occurrence of lymphocytopenia and its possible role as a marker for pandemic influenza A/H1N1 2009 virus infection in children.

## Methods

### *Study children*

Data were collected retrospectively from children referred to the Infectious Disease Unit, Anna Meyer Children's Hospital, Department of Sciences for Woman and Child's Health, University of Florence, between July 1<sup>st</sup> and November 1<sup>st</sup>, 2009 presenting with influenza-like symptoms (defined as a clinical picture compatible with influenza, e.g. sudden onset of disease, cough, fever > 38°C, myalgia and/or headache (Aguileira et al, 2003)). Inclusion criteria were 1) a PCR assay for pandemic influenza A/H1N1 2009 virus on a nasopharyngeal swab performed at admission, 2) simultaneous availability of WBC count data. Children with associated bacterial pneumonia, presenting with sore throats and positive strep test, those with detected IgM antibodies to Epstein-Barr virus and those with an underlying chronic condition (i.e.: severe congenital cardiopathy, immunodeficiency or malignancy) were excluded from the analysis.

Ethical approval for the study was granted from the Local College of Medicine Research

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3 Committee, Anna Meyer Children's University Hospital, Florence, Italy. The informed consent from  
4  
5 the children's parents/tutors was not required since the data collected remained anonymous.  
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8 *Laboratory investigations.*

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10 WBC count was performed by an automated analyser. C-reactive protein (CRP) was estimated by a  
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12 particle enhanced turbidimetric immunoassay (Flex<sup>®</sup> Reagent Cartridge, Dimension<sup>®</sup>, Siemens  
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14 Healthcare Diagnostics Inc., UK). Pandemic influenza A/H1N1 2009 virus infection diagnosis was  
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16 based on the results of a qualitative real-time PCR assay, performed on nasopharyngeal swabs,  
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18 according to the Centres for Disease Control and Prevention recommendations, as previously  
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20 described (Interim Recommendations for Clinical Use of Influenza Diagnostic Tests During the  
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22 2009-10 Influenza Season, September 29, 2009. website:  
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27 [http://www.cdc.gov/h1n1flu/guidance/diagnostic\\_tests.htm](http://www.cdc.gov/h1n1flu/guidance/diagnostic_tests.htm), accessed November 16th, 2009; Galli  
28  
29 et al., 2010).  
30

31 *Statistical analysis*

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33 Age, WBC count and differential cell count, red blood cell count, CRP levels, haemoglobin  
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35 concentration, platelet count and time elapsed between onset of disease and PCR assay were  
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37 expressed as median and interquartile range (IQR). In children, lymphocyte count results should be  
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39 interpreted on the basis of age-related reference values (Weerkamp et al., 2005). The z-score of  
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41 individual lymphocyte count indicates how much each absolute lymphocyte measurement varies  
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43 from the mean of the reference group in each age class, and may be useful to interpret study results  
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45 in children of different ages (Pembrey et al., 2008). Thus, the z-score of individual absolute  
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47 lymphocyte count, referring to reference values for each age class, was calculated (Nicholson et al.,  
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49 2003).  
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55 Receiver operating characteristic (ROC) curve analysis was conducted to determine the best  
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57 absolute lymphocyte count and lymphocyte count z-score thresholds in determining the presence of  
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59 positive RT-PCR assay, and corresponding sensitivity and specificity were reported. The area under  
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the ROC curve (AUC) with 95% confidence interval (CI) was also calculated.

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3 In order to assess the difference in proportions between groups, the  $\chi^2$  test or the Fisher test were  
4 used where appropriate. The Mann-Whitney-U test was used to compare continue variables.  
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6 Statistical analysis was performed using the statistical software SPSS for Windows, version 14.0. *P*  
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8 <0.05 was considered statistically significant.  
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## 11 12 **Results**

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14 Among 76 children enrolled into the study, 37 (48.7%) had a positive RT-PCR assay for pandemic  
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16 influenza A/H1N1 2009 virus in nasopharyngeal swab. The characteristics of these children are  
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18 reported in Table I.  
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21 When comparing data from these latter children with that from 39 children with uncomplicated flu-  
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23 like illness and negative pandemic influenza A/H1N1 2009 virus PCR, no difference in disease  
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25 duration, median age, red blood cell count, haemoglobin concentration, CRP values, and absolute  
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27 neutrophil count was observed, whereas significant differences were observed with respect to WBC  
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29 count, absolute and relative lymphocyte counts, absolute lymphocyte count z-score, and platelet  
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31 count (Table II). ROC analysis revealed that the best cut-points for absolute lymphocyte count and  
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33 absolute lymphocyte count z-score were 2,256/ $\mu$ L and -0.89, respectively, sensitivity being 0.81  
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35 (95%CI: 0.68-0.94), specificity 0.87 (95%CI: 0.77-0.98), positive predictive value 0.85 (95%CI:  
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37 0.74-0.97), and negative predictive value 0.83 (95%CI: 0.71-0.94). Absolute lymphocyte count and  
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39 respective z-score below the abovementioned cut-offs were observed in 30/37 (81.1%) infected  
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41 children *vs.* 7/34 (20.6%) controls ( $P < 0.0001$ ). (Figure 1).  
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## 48 **Discussion**

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50 The present study based on children with flu-like illness examined during a pandemic period, a  
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52 significantly lower median absolute lymphocyte count and absolute lymphocyte count z-score were  
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54 observed in children with documented pandemic influenza A/H1N1 2009 infection compared to  
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56 controls.  
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60 Lymphocytopenia was been previously documented in 61% of adult patients with influenza  
A/H1N1 2009 during the late March 2009 outbreak in Mexico (Perez-Padilla et al., 2009). Other

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3 authors reported lymphocytopenia as a possible marker for pandemic influenza A (H1N1) virus  
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5 infection in adults (Cunha et al., 2009; Coşkun et al., 2010). In one study, relative lymphocytopenia  
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7 (defined as <21% of WBC count) was present in approximately 90% of adults, but only in 3/16  
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9 (18.7%) of infected children (Cunha et al., 2009). The authors concluded that, although useful in  
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11 adults, lymphopenia appeared not to be a marker for pandemic influenza A/H1N1 2009 virus  
12  
13 infection in children. By contrast, lymphocytopenia (defined as an absolute lymphocyte count <  
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15 3000/ $\mu$ L) was observed in 91% of Chinese children with pandemic influenza A/H1N1 2009  
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17 infection (Cao B et al., 2009).  
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22 The present data not only confirm that lymphocytopenia is found commonly in children infected  
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24 with pandemic influenza A/H1N1 2009 virus infection, but also provide evidence that practical cut-  
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26 off points (absolute lymphocyte count <2,556 cells/ $\mu$ L or z-score <-0.86) may help to discriminate  
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28 children at higher risk of infection with 81% sensitivity and 87% specificity.  
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32 The finding of lower median platelet count in children with pandemic influenza A/H1N1 2009 virus  
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34 infection compared to controls has been previously observed (Cunha et al., 2009). However, in  
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36 both groups of children, platelet count was within the normal reference values and no child  
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38 displayed thrombocytopenia. Platelet count therefore appears not to be a useful marker for  
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40 pandemic influenza A/H1N1 2009 infection in children.  
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44 A potential limitation of the study described above is that no other specific virologic assay other  
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46 than PCR for pandemic influenza A/H1N1 2009 virus was performed, not allowing for specific  
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48 diagnosis in children flu-like symptoms but negative for pandemic influenza A/H1N1 2009 virus  
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50 PCR. This kind of analysis was however not the objective of this study. It is plausible theoretically  
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52 that false negatives may have been generated in a minor proportion of children with negative  
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54 influenza A/H1N1 2009 PCR assay. Nevertheless, the qualitative real-time PCR assay employed  
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56 here, and performed according to the Centres for Disease Control and Prevention  
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58 recommendations, has been demonstrated to be both highly sensitive and very specific (Interim  
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60 Recommendations for Clinical Use of Influenza Diagnostic Tests During the 2009-10 Influenza

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3 Season, September 29, 2009. website: [http://www.cdc.gov/h1n1flu/guidance/diagnostic\\_tests.htm](http://www.cdc.gov/h1n1flu/guidance/diagnostic_tests.htm),  
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5 accessed November 16th, 2009). Finally, due to the limited dataset results should be interpreted  
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7 with a degree of caution.  
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10 In conclusion, the data strongly suggest that lymphocytopenia may be used as a marker for  
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12 influenza A/H1N1 2009 infection in children, suggesting that it may help to discriminate children  
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14 with higher risk for infection during epidemics. From a practical point of view, absolute lymphocyte  
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16 count  $<2,556$  cells / $\mu\text{L}$  or z-score  $< -0.89$  may be useful cut-offs for adoption in children. These  
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18 findings have significant practical implications for the near future, in view of the fact that that the  
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20 pandemic virus is highly likely to continue to circulate in the next winter season (European Centre  
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For Peer Review

**Table I.** White blood cell count, relative and absolute lymphocyte counts and absolute lymphocyte count z-score, calculated considering age-related reference values (6), in 37 children referring to the Infectious Disease Unit, Department of Paediatrics, Florence University, Italy, with pandemic influenza A (H1N1) infection between July and November 2009.

Case identifier	Age (years)	WBC* count (cells/ $\mu$ L)	Relative lymphocyte count (% of WBC)	Absolute lymphocyte count (cells/ $\mu$ L)	Absolute lymphocyte count z-score
1	0.08	10,320	74.0	7,637	0.66
2	0.08	8,200	10.8	886	-1.62
3	0.08	6,250	35.2	2,200	-1.18
4	0.08	2,710	18.9	512	-1.75
5	0.08	4,700	19.2	902	-1.61
6	0.17	7,280	60.1	4,375	-0.44
7	0.17	15,510	36.9	5,723	0.01
8	0.33	9,220	34.6	3190	-0.84
9	0.50	6,680	15.5	1,035	-1.57
10	0.92	9,720	35.5	3,451	-0.75
11	2	11,950	21.1	2,521	-0.88
12	3	9,440	41.7	3,937	-0.07
13	4	4,760	46.7	2,223	-1.05
14	5	10,300	15.6	1,607	-1.41
15	6	5,150	10.7	551	-2.01
16	6	5,290	35.8	1,894	-1.24
17	6	12,640	6.7	847	-1.84
18	8	2,850	12.4	353	-2.12
19	9	6,330	14.2	899	-1.81
20	11	4,750	14.3	679	-1.94
21	11	4,110	20.4	838	-1.85
22	12	6,230	29.9	1,863	-1.26
23	12	5,940	29.7	1,764	-1.32
24	12	5,860	11.1	651	-1.95
25	12	8,280	6.1	505	-2.04
26	12	8,440	5.4	456	-2.07
27	13	2,320	25.6	594	-1.99
28	13	6,220	14.2	883	-1.82
29	13	3,710	33.6	1,246	-1.61
30	13	7,040	13.0	915	-1.80
31	13	19,710	59.3	11,688	4.37
32	14	8,180	8.6	704	-1.92
33	14	5,190	26.1	1,354	-1.55
34	14	6,010	31.3	1,881	-1.25
35	16	6,990	5.4	377	-2.11
36	16	5,600	20.2	1,131	-1.68
37	16	13,100	5.9	773	-1.88

Note. WBC: White blood cell count.

**Table II.** Data from 76 children with flu-like illness with or without pandemic influenza A (H1N1) infection.

	<b>Children with Influenza A H1N1 infection (n=37)</b>	<b>Children with viral respiratory infection (n=39)</b>	<b>P</b>
Males (n;%)	20 (54.1%)	18 (46.2%)	0.491
Age* (years)	9.0 (0.3-13.0)	9.1 (1.1-14.2)	0.896
Time period*between disease onset and influenza PCR assay (days)	1.9 (1.2-3.7)	1.7 (1.5-4.2)	0.641
WBC count* (cells/ $\mu$ L)	6,330 (5,170-9,330)	9040 (7450-11100)	0.002
Relative lymphocyte count* (%)	20.2 (11.7-34.9)	47.7 (33.8-56.1)	<0.0001
Absolute lymphocyte count* (cells/ $\mu$ L)	1,035 (691-2,211)	3,751 (2884-4832)	<0.0001
Absolute lymphocyte count z-score*	-1.61 (-1.90 -1.11)	-0.30 (-0.67 0.02)	<0.0001
Relative neutrophil count* (%)	60.8 (47.4-74.6)	40.8 (27.6-55.6)	<0.0001
Absolute neutrophil count* (cells/ $\mu$ L)	4,155 (2,721-5,770)	3,549 (2,470-5,194)	0.427
Red blood cell count* (1,000 cells/ $\mu$ L)	4,540 (3,840-4,945)	4,745 (4,445 -5,140)	0.062

Haemoglobin concentration* (g/L)	12.5 (11.0-14.0)	12.6 (11.8.-1350)	0.629
Platelet count* (1,000/ $\mu$ L)	252 (207-346)	338 (286-431)	0.003
C reactive protein* (mg/dL)	0.66 (0.34-1.80)	0.62 (0.40-1.29)	0.817

Note: \* *Median and interquartile range*

For Peer Review

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3 Legend to figure 1  
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6 A receiver operator characteristic (ROC) plot is shown, illustrating sensitivity and specificity of  
7  
8 absolute lymphocyte count and absolute lymphocyte count z-score in predicting pandemic influenza  
9  
10 A (H1N1) infection in 76 children with influenza-like illness during a pandemic period. Area under  
11  
12 the ROC curve was 0.852 (95%IC: 0.756-0.948) considering absolute lymphocyte count and 0.872  
13  
14 (95%IC: 0.783-0.961) for absolute lymphocyte count z-score;  $P < 0.0001$  vs. the identity line  
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16 (diagonal line).  
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For Peer Review