Potentially-toxic and essential elements profile of AH1N1 patients in Mexico City

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During spring of 2009, a new influenza virus AH1N1 spread in the world causing acute respiratory illness and death, resulting in the first influenza pandemic since 1968. Blood levels of potentially-toxic and essential elements of 40 pneumonia and confirmed AH1N1 were evaluated against two different groups of controls, both not infected with the pandemic strain. Significant concentrations of potentially-toxic elements (lead, mercury, cadmium, chromium, arsenic) along with deficiency of selenium or increased Zn/Cu ratios characterized AH1N1 cases under study when evaluated versus controlled cases. Deficiency of selenium is progressively observed from controls I (influenza like illness) through controls II (pneumonia) and finally pneumonia -AH1N1 infected patients. Cases with blood Se levels greater than the recommended for an optimal cut-off to activate glutathione peroxidase (12.5 μg/dL) recovered from illness and survived. Evaluation of this essential element in critical pneumonia patients at the National Institutes is under evaluation as a clinical trial.

In April 2009, a novel swine-origin influenza A (H1N1) virus was identified in patients from Mexico and North America. The new virus spread around the world and the World Health Organization (WHO) declared a pandemic level of 6. Since then, several publications presenting clinical and epidemiological characteristics and discussing risk factors associated to the H1N1 patients in Mexico have been published2–4. Mexico documented the largest number of confirmed cases during the first and subsequent outbreaks as documented in literature5. In this work we first documented major potentially-toxic elements concentration in 40 cases of pneumonia and confirmed AH1N1 were evaluated against two different groups of controls, both not infected with the pandemic strain. Significant concentrations of potentially-toxic elements (lead, mercury, cadmium, chromium, arsenic) along with deficiency of selenium or increased Zn/Cu ratios characterized AH1N1 cases under study when evaluated versus controlled cases. Deficiency of selenium is progressively observed from controls I (influenza like illness) through controls II (pneumonia) and finally pneumonia -AH1N1 infected patients. Cases with blood Se levels greater than the recommended for an optimal cut-off to activate glutathione peroxidase (12.5 μg/dL) recovered from illness and survived. Evaluation of this essential element in critical pneumonia patients at the National Institutes is under evaluation as a clinical trial.

Results

Participants of the study. Participants are divided in two main groups: Cases and Controls (I and II). The first group is referred to 40 AH1N1 cases (pneumonia hospitalized cases with confirmed viral infection through RT-PCR assay). Sample collection for this group was done from October 1st to December 31st 2009 and from January 15th to April 15th 2010 during pandemic year-period6. The second group is subdivided as Controls I and II. The first group (Controls I) is referred to 30 pneumonia cases negative for the pandemic strain through RT-PCR assay. The second group is referred to 64 persons admitted, treated for ILI (Influenza like Illness) symptoms also negative for the specific virus strain (through RT-PCR assay). Criteria for selection as a “control case” was being a relative living with the patient at the time of the disease, exposed to the virus through direct contact with him/her for at least three days before his/her hospitalization. Controlled cases were selected for the present study according to the period of our key AH1N1 samples were collected, i.e., from October 2009 throughout April 2010. Inclusionary and exclusionary criteria for selection of the control groups are summarized in Table 1.

Admission features of AH1N1 cases and controls. Cases. The start time of symptoms and admission to hospital ranged from 7–12 days (mean: 9 days). All patients had fever, temperatures higher than 38.5°C, cough, respiratory distress and other clinical features described for Mexico City cases2. Sample for the present study
was taken between the time of admission and positive results on the virus strain (AH1N1) through RT-PCR assay (2 days) before receiving any medication for the illness. At the time of admission thirty-six (90%) of study patients had increased lactase dehydrogenase (DHL) levels ranged from 511–1585 IU per liter. Twenty patients (50%) presented lymphocytopenia (low-normal lymphocytes account <1,000 per cubic millimeter). Three patients presented elevated creatinine levels (2.32–3.45 mg per deciliter). Fifteen cases (38%) presented lower than referenced hemoglobin values (Hb ≤ 12 grams per deciliter). Eight cases were off threshold than referenced values in hematocrit (36%–52%). All selected cases with unknown history of occupationally toxic-metals exposure. Table 2 presents characteristics of AH1N1 cases selected for the study.

Controls I and II. The start time of symptoms and admission/diagnosis in the hospital was between 5–7 and 3–5 days for Controls I and II, respectively. Controlled cases presented identical symptoms to those featured for the key cases, negative for the pandemic strain, all recovered from illness. They presented unknown history of occupationally toxic-metals exposure and went through blood tests as part of the research protocol approved by the National Council of Science and Technology of Mexico (CONACyT-Reference 126657-2009-02) during pandemic year. Sample for control groups was taken between the time of admission and negative results on the RT-PCR assay (2 days) before receiving any medication for the illness. Main characteristics of Controls I and II are summarized and compared to AH1N1 cases in Table 2. Details of both groups are described in Methods Section.

Critical care cases: survivals and non-survival AH1N1 patients. Ten of our AH1N1 cases received tracheal intubation; seven of these not survived. There were a total of 8 non-survivals AH1N1 patients documented in this research. All of them corresponded to samples taken in 2009 (1st October to 31st December). None of the non-survival patients received 2009 AH1N1 or seasonal influenza or pneumococcal vaccination before illness and hospitalization. All of

Table 1 | Inclusionary and exclusionary criteria for selection of Controls –I and II during pandemic study

| Inclusionary criteria | AH1N1 | Controls-I | Controls-II |
|-----------------------|-------|------------|-------------|
| 1) 20-55 years old; living in Mexico City metropolitan area | 14/40 (35%) | 12/30 (40%) | 21/64 (32.8%) |
| 2) Relative (not by blood) | 41.34 ± 12.6 | 44.2 ± 7.97 | 32.46 ± 10.64 |
| 3) Admitted and treated for different reasons (pneumonia: Controls-I) | 19–55 | 20–55 | 20–55 |
| 4) Admitted and treated for different reasons (ILI symptoms; Controls-II) | 44/26 | 46/12 | 31/16 |
| 5) Range of Hemoglobin: 12.0 g/100 mL–16.0 g/100 mL | 15 (37.5%) | 4 (13%) | 35 (54.7%) |
| 6) Range of Hematocrit: 38–48% | 10 (25%) | 8 (27%) | 21 (32.8%) |
| 7) Segregated (%): 19–30 yr. | 15 (37.5%) | 4 (13%) | 35 (54.7%) |
| 8) Segregated (%): 31–45 yr. | 10 (25%) | 8 (27%) | 21 (32.8%) |
| 9) Segregated (%): 46–55 yr. | 15 (37.5%) | 18 (60%) | 8 (12.5%) |
| 10) Smokers’ number/total number (%) | 23 (57%) | 11 (37%) | 23 (36%) |
| 11) Vaccinations | 0/40 (0%) | 0/30(0%) | 1/64 (1.5%) |
| 12) Body Mass Index-number/total No. (%) | 0/40 (0%) | 0/30(0%) | 1/64 (1.5%) |
| 13) Lactase dehydrogenase-U/liter | 884 (511-1585) | 801 (350-2600) | 13.8 |
| 14) Lymphocyte count – per mm³ | 39.07 | 39.4 | 42.09 |
| 15) Hemoglobin - g dL-1 | 39.07 | 39.4 | 42.09 |
| 16) Hematocrit - % | 39.07 | 39.4 | 42.09 |
| 17) Diabetes Mellitus (type 2) | 2/40 (5%) | None | None |
| 18) Asthma | 1/40 (2.5%) | 1/30 | None |
| 19) Pre-existing conditions-number/total No. (%) | 1/40 (2.5%) | None | None |
| 20) Abnormal laboratory findings-number/total No. (%) | None | None | None |
| 21) Only AH1N1 cases | 37/40 (92.5%) | 20/40 (50%) | 15/40 (38%) |
| 22) Lactase dehydrogenase > 350 U/liter | 8/40 (20%) | 8/40 (20%) | 8/40 (20%) |
| 23) Lymphocyte count < 1000 per mm³ | 15/40 (38%) | 15/40 (38%) | 15/40 (38%) |
| 24) Hemoglobin ≤ 12 g dL⁻¹ | 15/40 (38%) | 15/40 (38%) | 15/40 (38%) |
| 25) Hematocrit (38%–52%) | 15/40 (38%) | 15/40 (38%) | 15/40 (38%) |

Table 2 | Characteristics of AH1N1 Cases and Control study groups (Control I and II)

| Variable | AH1N1 | Controls-I | Controls-II |
|----------|-------|------------|-------------|
| Female sex-number/total No. (%) | 14/40 (35%) | 12/30 (40%) | 21/64 (32.8%) |
| Age (years) | | | |
| Mean (±SD) | 41.34 ± 12.6 | 44.2 ± 7.97 | 32.46 ± 10.64 |
| Range | 19–55 | 20–55 | 20–55 |
| Median/IQR | 44/26 | 46/12 | 31/16 |
| Segregated (%) | | | |
| 19–30 yr. | 15 (37.5%) | 4 (13%) | 35 (54.7%) |
| 31–45 yr. | 10 (25%) | 8 (27%) | 21 (32.8%) |
| 46–55 yr. | 15 (37.5%) | 18 (60%) | 8 (12.5%) |
| Smokers’ number/total number (%) | 23 (57%) | 11 (37%) | 23 (36%) |
| Vaccinations | 0/40 (0%) | 0/30(0%) | 1/64 (1.5%) |
| Body Mass Index-number/total No. (%) | 0/40 (0%) | 0/30(0%) | 1/64 (1.5%) |
| < 25 | 14/40 (35%) | 12/30 (40%) | 21/64 (32.8%) |
| 25–29.9 | 13/40 (32.5%) | 25–30 (100%) | 25–30 (100%) |
| 30–39.9 (III) | 11/40 (27.5%) | None | None |
| Morbid (III) | 02/40 (5%) | None | None |
| Laboratory findings-median (range) | | | |
| Lactase dehydrogenase-U/liter | 884 (511-1585) | 801 (350-2600) | 13.8 |
| Lymphocyte count – per mm³ | 39.07 | 39.4 | 42.09 |
| Hemoglobin - g dL-1 | 13.8 | 13.9 | 14.4 |
| Hematocrit - % | 39.07 | 39.4 | 42.09 |
| Pre-existing conditions-number/total No. (%) | | | |
| Diabetes Mellitus (type 2) | 2/40 (5%) | None | None |
| Asthma | 1/40 (2.5%) | 1/30 | None |
| COPD | 1/40 (2.5%) | None | None |
| Abnormal laboratory findings-number/total No. (%) | Only AH1N1 cases | | |
| Lactase dehydrogenase > 350 U/liter | 37/40 (92.5%) | 20/40 (50%) | 15/40 (38%) |
| Lymphocyte count < 1000 per mm³ | 15/40 (38%) | 15/40 (38%) | 15/40 (38%) |
| Hemoglobin ≤ 12 g dL⁻¹ | 8/40 (20%) | 8/40 (20%) | 8/40 (20%) |
these cases had obesity in some extent (5 cases: type I; 1 case: type II; 2 cases: morbid). Only three of them had a history of current smoking, suspended at the time of presenting symptoms. Mean age of non-survival patients (8/40) of the study was 45.33 years.

**Multi-element determination in whole blood of AH1N1 cases and controls.** 28–31 metals and metalloids were accurately reproducible through evaluation of certified and referenced standards described in Methods Section. Results of the present study are focused on whole blood concentration levels of the following elements: lead, mercury, chromium, cadmium, arsenic and selenium, zinc, copper, calcium and sodium.

(a) **Potentially-toxic metals profile for cases and controls analysed.** Table 3 describes statistic parameters for concentration levels of lead, mercury, cadmium, arsenic and chromium in whole blood of cases and controls analysed. Table 4 presents statistical analysis results for lead and mercury heavy-metals. Details of statistics approaches applied to evaluate significant differences between cases and controls are depicted in Methods section.

**Lead (Pb).** Blood lead levels ranged from 43.77 to 366.18 ppb for AH1N1 cases and from 2.04 to 115.79 ppb for controls. Mean blood lead levels for cases were greater than literature2 values (62.8 ppb) 75% of the time (30/40 cases) while for Controls I and II the time, respectively. Mean blood lead levels for AH1N1 patients (62.8 ppb) 75% of the time (30/40 cases) while for Controls I and II, median values were almost twice (1.9) and five (4.8) times, than concentration levels found for this elements in the control group –II. Statistical analysis is not performed for Chromium as more than 50% of the values are within the lower detection limit (LDL) of the analytical technique, as detailed in Table 3.

**Cadmium (Cd).** Blood cadmium levels in AH1N1 patients ranged from 0.278 to 8.94 μg/dL; mean of 4.604 ± 1.94 SD μg/dL, greater than literature2 reported values. Cd (as well as Pb and As) determinations in samples of control group (I) were not achieved for this pandemic study. In spite of this, samples of cases and controls II analysed showed mean and median blood levels of AH1N1 patients were substantially higher, almost four (3.9) and five (4.8) times, than concentration levels found for this elements in the control group –II. Statistical analysis is not performed for Chromium as more than 50% of the values are within the lower detection limit (LDL) of the analytical technique, as detailed in Table 3.

**Mercury (Hg).** Mean blood mercury levels for cases (AH1N1) and Controls (I and I) were slightly higher than threshold referenced9–10 values (mean 1.1 μg/dL). As shown in Table 4, statistical analysis between blood mercury levels of pneumonia –infected AH1N1 patients versus Controls II (ILI cases) met highly statistical significance while between Controls I (pneumonia cases) and controls II did not. Probable reasons for Hg blood levels found among population living in Mexico City during pandemic influenza might be the content of mercury in airborne particulate matter recently reported in literature11. Mercury belongs to one of the most harmful xenobiotic with severe implications (e.g., kidney impairment) although chronic low-dose exposure remains still not well understood9.

**Chromium (Cr).** Chromium blood levels for AH1N1 patients ranged from 0.278 to 8.94 μg/dL; mean of 4.604 ± 1.94 SD μg/dL, greater than literature2 reported values. Cr (as well as Cd and As) determinations in samples of control group (I) were not achieved for this pandemic study. In spite of this, samples of cases and controls II analysed showed mean and median blood levels of AH1N1 patients were substantially higher, almost four (3.9) and five (4.8) times, than concentration levels found for this elements in the control group –II. Statistical analysis is not performed for Chromium as more than 50% of the values are within the lower detection limit (LDL) of the analytical technique, as detailed in Table 3.

| Table 4 | Potentially-Toxic Elements: Statistical analysis |
| --- | --- |
| **a) Kruskal Wallis test.** |
| Element | N | Kruskal Wallis Statistic | Degrees Freedom | p-value |
| Pb | 135 | 47.382 | 2 | 0.000 |
| Hg | 101 | 22.862 | 2 | 0.000 |
| **b) Multiple bivariate comparisons. Post Hoc Bonferroni correction.** |
| Element | Category Group | Statistic | p-value | p value Bonferroni correction 1 |
| Pb (ppb) | Cases vs. Control I | 39.257 | 0.000 | 0.000 |
| Cases vs. Control II | 53.472 | 0.000 | 0.000 |
| Cr vs. C I | 14.215 | 0.100 | 0.300 |
| Hg (μg/dL) | Cases vs. Control I | 15.329 | 0.060 | 0.181 |
| Cases vs. Control II | 30.940 | 0.000 | 0.000 |
| Cr vs. C II | 15.612 | 0.054 | 0.162 |

1Corresponds to 0.05 significance level.
an important limitation to evaluate statistical significance between subgroups.

**Arsenic (As).** Blood arsenic levels for AH1N1 patients ranged from 1 to 44 ppb; mean of 8.62 ± 12.27 SD ppb, remarkably greater than literature's values (range: 2.6–17.8 ppb; mean: 5.0 ppb). Indeed, seven of our eleven AH1N1 cases having the highest As concentrations (range: 11–36 ppb) did not recover from illness. Nevertheless, as seen in Table 4, more than 50% of the As concentrations of both cases and controls (II) were detected within the LDL resulting in an important limitation to evaluate statistical significance between subgroups.

(b) **Essential elements profile for cases and controls analysed.** Table 5 describes statistic parameters for concentration levels of selenium, zinc, calcium, sodium and copper in whole blood of cases and controls I and II. Table 6 presents statistical analysis results for selenium (Mann-Whitney/Bonferroni correction) and the rest of essential elements (Mann-Whitney U Tests). Statistics analysis approaches are detailed in Methods section.

**Selenium (Se).** Mean blood Se levels for AH1N1 was 10.62 μg/dL, versus controls I and II concentrations of 13.67 and 15.46 μg/dL, respectively; within reported values (range: 11.0 to 15.0 μg/dL). Overall blood selenium values in key AH1N1 cases tended to decrease versus controls I and II. This is in agreement with results showed in Table 6 where blood selenium levels of AH1N1 patients were statistically significantly lower when compared to both Controls I and II. It is also noted that selenium blood levels also reached statistical significance for Controls I (pneumonia) when compared to controls I (ILI).

Survival and non-survival AH1N1 cases. Cases with selenium concentrations greater than 12.5 μg/dL-level, suggested as the optimal cut-off for glutathione peroxidase activity recovered from illness in fewer days (5–7 days) than the average time (10–12 days for pneumonia; 15–21 days for pneumonia –AH1N1 infected ones) registered for cases analysed during pandemic year at INER. Seven (7/8) non-survival cases registered selenium levels lower than the optimal cut-off abovementioned. Four of critical patients presenting (7/8) non-survival cases registered selenium levels lower than the optimal cut-off abovementioned. Four of critical patients presenting

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### Table 5 | Essential Elements Profile: Descriptive Statistics

| Element | N | Min | Mean | SD | Median | IQR | Max |
|---------|---|-----|------|----|--------|-----|-----|
| Se (μg/dL) | Cases | 40 | 6.22 | 10.62 | 2.81 | 9.68 | 3.22 | 18.00 |
| | Controls I | 19 | 11.69 | 13.67 | 1.14 | 13.93 | 1.92 | 15.94 |
| | Controls II | 64 | 11.13 | 15.46 | 1.81 | 15.13 | 2.17 | 21.86 |
| Zn (μg/dL) | Cases | 40 | 282.26 | 669.48 | 260.01 | 597.52 | 320.01 | 1285.40 |
| | Controls II | 65 | 432.02 | 622.47 | 271.80 | 582.32 | 108.01 | 2148.10 |
| Ca (mg/dL) | Cases | 40 | 1.80 | 3.99 | 0.57 | 4.07 | 0.57 | 4.82 |
| | Controls II | 39 | 1.77 | 3.90 | 1.22 | 4.29 | 0.50 | 4.94 |
| Na (mg/dL) | Cases | 40 | 188.04 | 271.96 | 23.62 | 274.89 | 15.75 | 316.04 |
| | Controls II | 11 | 259.56 | 286.72 | 19.55 | 287.62 | 29.16 | 317.27 |
| Cu (μg/dL) | Cases | 40 | 64.34 | 111.55 | 23.05 | 108.29 | 27.18 | 163.74 |
| | Controls II | 24 | 102.55 | 126.63 | 28.61 | 112.78 | 26.25 | 207.99 |

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**Table 6 | Essential Elements: Statistical Analysis**

**a) Kruskal Wallis test.**

| Element | N | Se (μg/dL) | Kruskal Wallis Statistic | Degrees Freedom | p-value |
|---------|---|------------|--------------------------|----------------|---------|
| Cases   | 124 | 59.424 | 2 | 0.000 |

**b) Multiple bivariate comparisons. Post Hoc Bonferroni correction.**

| Element | Category Group | Mann Whitney p-value | Bonferroni correction1 |
|---------|----------------|---------------------|------------------------|
| Se (μg/dL) | Cases vs. Control I | 0.003 | 0.0081 |
| | Cases vs. Control II | 0.007 | 0.0211 |
| | C-I vs. C-II | 0.000 | 0.0001 |

**c) Mann-Whitney U test.**

| Element | p-value | Zn (μg/dL) | Ca (mg/dL) | Na (mg/dL) | Cu (μg/dL) |
|---------|---------|------------|------------|------------|------------|
| Cases vs. Control II | 0.853 | 6.283 | 0.027 | 0.074 | 0.023 |

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1Corresponds to 0.05 significance level.
2Hodges-Lehman.
when compared with WB levels of pneumonia and ILI controls, both
clinically significant (P < 0.001) and remarkably (Cd, Cr, As) different
Discussion
when a pre-condition (particularly, obesity III) occurred. Number
between survivals and non-survivals AH1N1 cases were observed
(morbid).

64.34 to 163.74
Copper (Cu).

111.55 and 127 µg/dL for controls and controls II, respectively). It is noted

0.001) lower (mean: 10.42 µg/dL) than controls I and II (mean 13.67; 15.19 µg/dL, respectively). It is noted highly statistically significant decrement of selenium from controls II

survival. Nevertheless the effect of selenium status on responses to viral infection is not fully understood, selenium
depletion has been shown in a cell culture model to affect influenza
virus-induced cytokine production in bronchial epithelial cells29.
With regards to renal failure (non-survival cases), it should be noted
that this condition further decreases Se levels and glutathione per-
oxidases in blood30. Based on clinical-lab features of ICU patients
who survived from those who did not, evaluation of key nutrients,
such as selenium, is currently under evaluation in the recovery of
pneumonia -viral -infected patients at the National Institute for
Respiratory Diseases. Finally, increased Zn/Cu ratios were observed in
AH1N1 patients versus controls not infected with the pandemic
strain. Furthermore, Zn/Cu ratios were remarkably high for AH1N1
cases where obesity (category II-III) was a pre-existing condition.


Environmental exposure to lead through airborne particulate matter
might be considered with reserves as leaded gasoline was phase-out
from Mexico by federal and local stricter regulations on this matter
since 199727. On the other hand, potentially-toxic blood levels of at
least three of the five heavy-metals studied (Pb, Cd, Cr) were remark-
ably higher in AH1N1 -infected patients segregated by smoking
failure14,15. Nevertheless, statistical analysis was not conclusive on
this matter (inhomogeneity of different subgroups), particularly in
the context of severity of the illness (survival or non-survival
patients), at least for cases under study. Along with significant con-
centrations of potentially-toxic elements, deficiency of some essen-
tial metals (Se, Cu) characterized AH1N1 viral-infection cases in
Mexico City. WB levels of selenium in AH1N1 patients remained
statistically significantly (p<0.001) lower (mean: 10.42 µg/dL) than
controls I and II (mean 13.67; 15.19 µg/dL, respectively). It is noted highly statistically significant decrement of selenium from controls II
(influenza like illness) through controls I (pneumonia) and finally in
pneumonia -AH1N1 infected patients, the latter registering the low-
est values of the essential element. Selenium values for critical (ICU)
patients who survived from those who did not, capture our attention.
Results showed that all AH1N1 patients having blood Se levels
higher than the recommended level for an optimum—i.e., to activate
glutathione peroxidase (12.5 µg/dL) recovered from the illness in a
shorter time and survived. Nevertheless the effect of selenium status
on responses to viral infection is not fully understood, selenium
depletion has been shown in a cell culture model to affect influenza
virus-induced cytokine production in bronchial epithelial cells29.

Differences in blood copper levels
Survival and non-survival patients. There were no substantial differ-
ces in blood Ca levels between survivals and non-survivals for
AH1N1cases analysed here.

Calcium (Ca).

Blood calcium levels for cases ranged from 1.8 to 4.82
go/dL; slightly below literature values (300–320 mg/dL). Calcium concentrations
were not achieved for samples of controls-I as aforementioned. No
statistically significant differences were observed when compared
calium blood levels of cases versus controls II, as shown in
Table 6. Nevertheless, lesser calcium values (30% lower than mean
values) corresponded 60% of the time with higher lead values (≥14–
15 µg/dL) for AH1N1 cases under study.

Survival and non-survival patients. There were no substantial differ-
ces in blood Zn levels between survivals and non-survivals for
AH1N1cases analysed here.

Zinc (Zn). Blood zinc levels for AH1N1 patients ranged from
6.34 to 163.74 µg/dL and to 208 µg/dL for controls II. Mean
values of 111.55 and 127 µg/dL for cases and controls -II, respect-
ively, both within literature (80–140 µg/dL). Copper concentrations
were not achieved for samples of controls -I as aforementioned.
Significance was achieved for Cu concentrations levels between
AH1N1 cases and controls -II as shown in Table 6.

A metabolic imbalance is observed when deficiency of the essential
element resulted in higher ratios of zinc to copper for AH1N1 influ-
enza cases under study (6.07) versus their controls (5.52). It is also
noted that Cu blood levels in AH1N1 patients tended to decrease
(arithematically different) with obesity, in agreement with previous studies27. For AH1N1 cases ana-
lysed in this research this was particularly observed for category III
(morbid).

Survival and non-survival patients. Differences in blood copper levels
between survivals and non-survivals AH1N1 cases were observed
when a pre-condition (particularly, obesity III) occurred. Number
of cases to evaluate significance between copper levels was a limita-
tion to perform statistical analysis between these two subgroups.

Discussion
We have showed that whole blood (WB) profile of potentially-toxic
heavy metals in pneumonia -AH1N1 infected patients was statist-
ically significant (Pb, Hg) and remarkably (Cd, Cr, As) different
when compared with WB levels of pneumonia and ILI controls, both
negative for the pandemic strain. Higher WB lead concentrations
(≥14–15 µg/dL) for cases under study were related (in 38% of the
cases) to a lower than referenced values of hemoglobin; lesser con-
centrations of Ca2+ and Zn2+ (60–62% of the time) were registered
for aforementioned concentrations as well. Substitution of essential
elements by lead has been widely mentioned in literature though
underlying mechanisms have not been fully elucidated28. Spite the
fact that key cases and controls were selected accordingly to specific
guidelines of the research protocol regarding "no exposure to a spe-
cific environmental source of toxicity related to the metals and metal-
loids considered in the study", concentration levels of some
potentially-toxic metals (e.g. Pb) found in this study might be, in
part, caused by an unknown source determined by environmental
conditions of Mexico City inhabitant’s resident such as legacy of past
domestic uses (leaded paints or ceramics) as well as environmental
exposure to dust and soil remissions containing heavy-metals22.

Methods
Study participants. AH1N1 patients. The pandemic influenza study was conducted at INER as part of a national public health investigation during the collection of
AH1N1 samples and review of clinical data (2nd semester 2009-1st semester of 2010). Selection of the key AH1N1 cases was done accordingly to standards and
specifications of the research protocol approved by the National Council of Science
and Technology in Mexico (CONACyT-Reference 126657) in 2009. The pandemic
influenza study was also approved by the Committee of Science and Bioethics of
the National Institute of Respiratory Diseases in the same year. All patients selected had
pneumonia and positive result on reverse-transcriptase-polymerase-chain-reaction

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pneumonia and positive result on reverse-transcriptase-polymerase-chain-reaction

Survival and non-survival patients. There were no substantial differ-
ces in blood Na levels between survivals and non-survivals for
AH1N1cases analysed here.

Sodium (Na).

Blood sodium levels for key cases ranged from 188.04
to 316.04 mg/dL; mean value 271.95 mg/dL. Blood sodium levels for
controls –II ranged from 260–317 mg/dL; mean value of 286.72 mg/
dL; slightly below literature values (300–320 mg/dL). Sodium concentra-
tions were not achieved for samples of controls -I as aforementioned.
No statistically significant differences were observed when compared
sodium blood levels of cases versus controls II, as shown in
Table 6. Nevertheless, lesser sodium values (30% lower than mean
values) corresponded 60% of the time with higher lead values (≥14–
15 µg/dL) for AH1N1 cases under study.

Survival and non-survival patients. There were no substantial differ-
ces in blood Na levels between survivals and non-survivals for
AH1N1cases analysed here.

Copper (Cu). Blood copper levels for AH1N1 patients ranged from
64.34 to 163.74 µg/dL and to 208 µg/dL for controls II. Mean
values of 111.55 and 127 µg/dL for cases and controls -II, respect-
ively, both within literature (80–140 µg/dL). Copper concentrations
were not achieved for samples of controls -I as aforementioned.
Significance was achieved for Cu concentrations levels between
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enza cases under study (6.07) versus their controls (5.52). It is also
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(arithematically different) with obesity, in agreement with previous studies27. For AH1N1 cases ana-
lysed in this research this was particularly observed for category III
(morbid).

Survival and non-survival patients. Differences in blood copper levels
between survivals and non-survivals AH1N1 cases were observed
when a pre-condition (particularly, obesity III) occurred. Number
of cases to evaluate significance between copper levels was a limita-
tion to perform statistical analysis between these two subgroups.
(RT-PCR) testing for influenza A (H1N1) according to guidelines from the U.S. Center for Disease Control and Prevention (CDC) published during pandemic influenza24. All participants received Institutional informed written consent also approved by Institutional Committee of Bioethics and Science at INER, before the sample collection and laboratory tests.

Control subjects. Two groups of controls were selected for purposes of our study. Controls -I corresponded to thirty (30) subjects admitted and diagnosed with pneumonia but negative for the pandemic strain. Controls –II corresponded to 64 subjects directly exposed to the AH1N1 virus in Mexico City diagnosed as influenza pneumonia but negative for the pandemic strain. They were selected according to the age range of influenza patients (20–55 years), having unknown history of occupational toxic exposure, and with any pandemic year. They were selected accordingly to guidelines detailed in the research protocol aforementioned. Sample collection of controls initiated during first outbreak and extended throughout WHO pandemic year. They were selected according to the age range of influenza patients (20–55 years), having unknown history of occupational toxic exposure, and with any influenza (AH1N1 or seasonal) or pneumococcal vaccines previously to blood sample was taken. Criteria for selection of the control during pandemic period was being one of the closest AH1N1 patient’s relatives (not by blood), exposed to the virus through direct contact with him/her for at least three (3) days before patient’s hospitalization. All participants received medical consultation, blood (biometry and biochemistry) tests accordingly to the CONACYT research protocol and were selected having unknown history of pre-existing health conditions. Controlled cases received Institutional informed consent and also approved by the Committee of Bioethics and Science at INER, before the sample collection and laboratory tests. Both INER and INCNMSZ are National Public Hospitals that serve a low-middle social strata population mainly from Mexico City and its Metropolitan Area. For inclusionary and exclusionary criteria of selection as a “control” for pandemic influenza research protocol, refer is Table 1.

Clinical-lab data. Clinical-medical charts of the 40 patients hospitalized for pneumonia at INER were reviewed. Real-Time Polymerase Chain Reaction (RT-PCR) of all patients was positive for AH1N1 at admission though nasopharyngeal-swab sample or after tracheal intubation for Intensive Care Unit (ICU) cases. All procedures for RT-PCR assay were followed according guidelines provided by the U.S. CDC in 2009. Age, gender, history of smoking, diabetes mellitus or other pathologies, body mass index (BMI) as well as laboratory clinical levels of serum lactic dehydrogenase (DHL), leukocyte counts, creatinine, hemoglobin and hematocrit levels at admission were compiled in the clinical-lab database for the study.

Blood sample collection. Trained medical and research staff performed all sample collection at the National Institutes (INDER and INCNMSZ) in Mexico City, Mexico. Original plus replicate whole-blood samples of 3–5 ml were collected in metal-free tubes for multi-elemental trace element analyses. All whole-blood collection and processing materials were acid cleaned, according to Mexican Norms. All reagents were ultra-high purity or ultrapure analytical grade. Selected samples for the protocol were frozen at -20°C at the INCNMSZ until chemical analyses, according to sample collection guidelines for trace elements in blood28–31.

Trace elements quantification. 28–31 metal and metalloid determination were quantified from 60 acid-digested whole blood samples (AH1N1 patients’ samples plus blanks (determinations) through an Inductively-Coupled Plasma Mass Spectrometer (ICP-MS; Agilent Technologies, U.S.) and an Atomic Absorption Spectrometer (AAS; Perkin Elmer, U.S.) at Laboratory of Instituto de Ciencias del Mar y Limnología from the National Autonomous University of Mexico (UNAM). Elements determined through ICP-MS were Li, Mg, Al, K, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ge, As, Se, Sr, Y, Mo, Ag, In, Sn, Sb, Ba, Ti, Pb, Hg and Bi. Lead and zinc quantifications were double-verified by AAS– graphite furnace; technique that was also applied for cadmium determination. Mercury was determined by AAS– hydride generation. Finally, calcium and sodium measurements in whole blood were performed by AAS-flame. The three variants (flame, hydride generation and graphite furnace) in AAS are literature recommended techniques for quantification of metals and metalloids of interest. The CONACyT research protocol was also subject to external quality control on the elemental analysis to verify ICP-MS and AAS determinations of the core (blood) samples of AH1N1 patients through an Inductively Coupled Plasma- Collision Cell- Mass Spectrometer (ICP-CC-MS) at the Instituto de Geología from the Autonomous University of San Luis Potosí (UASLP), San Luis Potosí, Mexico. As a target of quality control program and the inter-comparison of internal and external laboratories we applied same certified referenced materials from the International Atomic Energy Agency (IAEA-A-13 freeze dried animal blood) and High Purity CRM (Bovine Liver) to evaluate accuracy and reproducibility of the analytical methods performed. ICP-MS determination of AAS literature recommended technique had here an adequate linearity, accuracy and precision during the quantification study. Briefly, the method yields a measurement precision of ± 0.5%RSD for lead (reference potentially-toxic element) concentrations of <0.003 μg/ml. Trace-elements from control samples were also double-verified by ICP-CC-MS following the same ICP-MS methodology and certified standards. The analytical detection limit determined was 0.1 μg/l for ICP-CC-MS and 1–2 μg/l for AAS. Metal and metalloid concentrations are reported in micrograms per deciliter (μg/dl) or micrograms per liter (ppb), unless otherwise indicated. Statistical Analysis. Non-parametric analysis was applied to significant differences on concentrations of potentially-toxic elements and essential elements in whole blood of AH1N1 patients versus their controls. This variant was chosen as at least one assumption, e.g. independence and normality or homogeneity of variances was not adequate to perform one-way ANOVA analysis. Kruskal-Wallis test was applied to evaluate significant differences concentrations of potentially-toxic elements lead and mercury and essential element selenium in whole blood of AH1N1 patients versus their controls and between their respective controls (II). Data reported in the present study is expressed as mean ± SD (standard deviation), median and IQR (inter-quartile range). Significance was set as p<0.05; highly statistical significance was considered as p<0.001.

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Author contribution
M.M. conceived and directed the toxicology AH1N1 study and wrote the paper. E.G.B. evaluated clinical aspects of critical and non-critical patients. A.V.-G. evaluated medical and laboratory blood tests for the collection and selection of blood samples for the research. G.T. performed statistical analysis. F.V.G. designed the protocol for the pre-treatment of viral-infected samples at UNAM. F.V.G. & M.E.G.-A. quantified toxicology and essential multi-element profiles in blood samples of AH1N1 and controls. M.C. & A.H. (Epidemiology) helped with controlled cases.

Additional information
Competing financial interests: The authors declare no competing financial interests.
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