British Journal of Medicine & Medical Research
5(4): 480-498, 2015, Article no.BJMMR.2015.053
ISSN: 2231-0614

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Comparing the Metal Concentration in the Nails of Healthy and Cancer Patients Living in the Malwa Region of Punjab, India with a Random European Group – A Follow up Study

Eleonore Blaurock-Busch[1,2*], Yvette M. Busch[1,2], Albrecht Friedle[3], Holger Buerner[1,2], Chander Parkash[4] and Anudeep Kaur[4]

[1]*Research Department, Trace Minerals International, Colorado, USA.*
[2]*Research Department, Micro Trace Minerals, Hersbruck, Germany.*
[3]*Analytical Department, Labor Friedle GmbH, Regensburg, Germany.*
[4]*Biology Department, Punjab Technical University, Kapurthala, Punjab, India.*

Authors' contributions

This work was carried out in collaboration between all authors. Author EBB was responsible for the coordination of the overall study, including: the study design, data analysis and manuscript preparation. Author CP managed collaboration amongst investigators, sample collection and patient survey. Author YMB managed sample coordination; author AF managed sample testing and data analysis. Author HB managed manuscript review, literature searches and data analysis. Authors CP, AK and YMB managed staff training and manuscript review. All authors were involved in study design and management.

Article Information

DOI:10.9734/BJMMR/2015/13124
Editor(s):
(1) Anonymous
Reviewers:
(1) Anonymous, Institute of Chinese Medicine, The Chinese University of Hong Kong, Hong Kong.
(2) Peizhong Mao, Physiology and Pharmacology, Oregon Health and Science University, Beaverton, USA.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=663&id=12&aid=6192

Original Research Article

Received 4th August 2014
Accepted 23rd August 2014
Published 23rd September 2014

ABSTRACT

The cancer prevalence in the Malwa region of Punjab (1089/million/year) is much higher than the national average cancer prevalence in India (800/million/year). In our previous study on hair metal analysis, we located a high metal burden in Punjabi cancer patients and their live-in relatives, suggesting that an excessive metal exposure is a factor in the pathogenesis of cancer. The present study focused on nail metal analysis, a biological material similar to hair. Previously, we had used

**Corresponding author: Email: ebb@microtrace.de;*

ICP-MS spectroscopy to confirm high exposures to aluminium (Al), barium (Ba), manganese (Mn), lead (Pb), uranium (U and other metals in the hair of Punjabi cancer patients and their healthy relatives (Blaurock-Busch et al. 2014). In this study, we used nail metal analysis to confirm the results of our previous study. We compared the nail metal concentration of healthy Punjabis (N=83) with randomly selected healthy Europeans (N=83) and found highly significant differences between the European and Punjabi groups, including the healthy and the cancer groups.In comparison, our European group showed a low percentage (0 to 13%) of pathological values for aluminium (Al), barium (Ba), cadmium (Cd), iron (Fe), manganese (Mn), nickel (Ni), lead (Pb), strontium (Sr), titanium (Ti) and uranium (U), while the healthy Punjabi groups showed between 13% and 99% pathological values for these elements. (Explanation: A test value above the 95% reference range is considered a pathological value.)The greatest metal burden was found in the breast cancer group (N=13), showing 100% pathological values for Al, Fe, Mn and U. This study supports previous research, which demonstrated a significant metal burden in Punjabi people. Water, soil, and phosphate fertilizers may be the cause of this excessive exposure.

Keywords: Punjab; Malwa region; cancer; breast cancer; nail analysis; aluminium; iron; manganese; strontium; uranium.

1. INTRODUCTION

Punjab is one of the leading food grain producing states in India. It is also the region with the highest cancer rate. The cancer prevalence (per million per year) in the Malwa region is indicated to be 1089. In comparison, this is much higher as it is in two other regions of Punjab, *i.e.*, Majha (647/million/year) and Doaba (881/million/year). The national average cancer prevalence in India is 800/million per year. Four of the 11 districts in the Malwa region are most afflicted by various cancers: Muktsar, followed in order by Mansa, Faridkot and Bathinda [1].

Studies indicate that excessive use of mineral fertilizers and pesticides has led to water and soil contamination, affecting farming communities. In the Malwa region, cancer mortality is directly correlated with gender and farming (Singh, 2008) [2]. The indiscriminate use of pesticides, fertilizers, as well as poor groundwater quality is considered to be among the main reasons for the high incidence of several diseases in the Malwa belt. Farming communities have higher cancer rates [3]. Studies indicate that drinking water, particularly in the Malwa belt, can be a source of heavy metals, including fluoride and pesticides [4,5].

Toxic metals are considered to be contributory causes for a variety of cancers [6-8]. Exposure to cadmium (Cd) and other heavy metals is a well-known risk factor for cancer. Cd has been found in fruit and vegetables grown on contaminated soil [9]. Stoica et al. studied the effect of Cd on estrogen receptor levels and estrogen-induced responses in human breast cancer cells [10]. Compared to healthy breast tissue, previous studies discovered a significant presence of Cd in malignant breast cancer tissue [11-13].

We chose to test human nail samples, because just like hair, nails are body tissue. Nail metal analysis can be used as a diagnostic test to evaluate chronic metal exposure. In recent years the interest in nails as a biological sample for metal analysis has been growing [14-20].Rashad and Hossam from the Chemistry Department, South Valley University, Aswan, Egypt proved that ‘human scalp hair and fingernails could be used successfully as a biological indicator for the assessment of heavy metal pollution ’[21].

Nails and hair are metabolic end products that incorporate metals into their structure during the growth process [22,23]. Both specimen have been suggested as suitable measures of heavy metal pollution. Their advantage over the conventional blood metal analysis is that hair and nails reflect exposure and metal uptake of previous months, representing a longer integrator of heavy metal exposure [24]. The uptake of lead, for example, suggests a three compartmental tool for the lead metabolism, namely circulation in blood, accumulation in soft tissue with keratin and collagen being the target proteins [25].

Nails and hair have advantages over other biological materials [26,27]:

1. Samples can be obtained without injury to the donor.
2. Samples can be stored for a long time, before and after analysis without any changes

3. Higher concentrations of toxic metals are found in nail or hair samples when compared to blood or urine
4. Samples reflect past exposure and accumulation of metals over a long period of time

Of the 83 healthy Punjabis tested, 13% showed nail cadmium concentrations above the accepted reference range, in the healthy European population only 6% exceeded that range. In the healthy cancer relative group 9% of the test values were above the reference range; in the cancer group we noted 0% (Fig. 1). We suggest that redistribution of cadmium plays a role i.e. accumulation in cancer tissue as outlined above seems logical.

Chronic arsenic exposure is of concern to Indians and has been listed as a cause of a variety of cancers, including skin and several internal cancers. For the last decade, the alarming rate of cancer mortalities in many villages of Punjab has been associated with this toxic metal [2] and its presence in deep water wells and hand pump water. While WHO (World Health Organization) has set a maximum contaminant level of 10ppb for arsenic in drinking water, wells located in and around Amritsar showed arsenic concentrations of up to 85ppb [28].

Nails are considered reliable material to monitor arsenic exposure [29]. Mees' lines, a single transverse broad band of white discoloration of nails, are a well-known sign of arsenic poisoning [30]. We could not detect these signs in our nail samples. In our healthy Punjabi test group 4.8% of the as values exceeded the reference range, compared to 2.94% of the cancer group and 6% for the Cancer relative group. We found 0% in the European group.

In our previous Punjabi studies, we detected unusually high uranium concentration in urine and hair samples in children and adult populations. Our 2010 study involved physically and mentally handicapped Punjabi children [31]. We found pathological uranium levels in the hair of 77% of the adult population (N=149) and 88% in the handicapped children <12years of age (N=114), reflecting chronic exposure. Urine testing showed 42% pathological values for the children prior to any type of detoxification treatment, suggesting immediate exposure. In 2014, we tested hair uranium concentrations in Punjabi cancer patients and their healthy relatives. Of the cancer patients (N=50) 71% exceeded the reference range of 0.1mcg/g for uranium in hair compared to 58% of the healthy group of relatives tested (N=50).

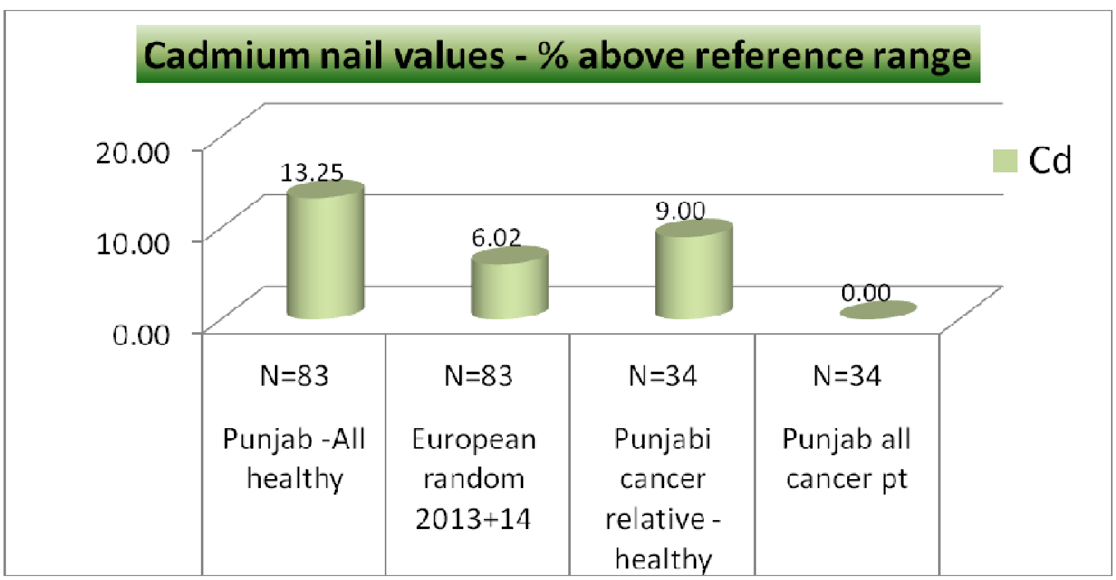

Fig. 1. Percent of pathological cadmium values in test groups

Note: A test value above the 95% reference range is considered a pathological value

A test value above the 95% reference range is considered a pathological value. Our present nail study showed 100% pathological values for U in the cancer group (N=34) and 100% for the group of healthy cancer relatives (N=34). When we compared the healthy European group (N=83) with a healthy Punjab population (N=83) we found significant differences (Table 1).

Uranium is a naturally-occurring radioactive element, commonly found in very small amounts in water, rock, soil or human tissue, and it contributes to low-level background radiation and various cancers. Long term chronic intakes of uranium in food, water, or air can lead to internal irradiation and/or chemical toxicity. Intakes exceeding EPA standards increase cancer risks, liver damage, or both [32].

A considerable discrepancy in pathological values for all the elements was noted between the European and the Punjabi healthy groups as shown in Table 1.

1.1 Participants

We first tested the nails of the same healthy individuals and cancer patients of Punjab that had previously been selected for our hair study on cancer [33]. All Punjabi test persons are from the Malwa region. All persons accepted into our study signed a consent form.

Nails samples had been collected along with the hair samples in 2012/2013. Of the original group of 50 healthy and 50 cancer patients, we were able to use the nail samples of 34 cancer patients and 34 of their healthy cancer relatives. Of the total cancer group (N=34), 13 were breast cancer patients. To verify national differences, we also compared nail results of 83 healthy Punjabis and 83 healthy Europeans. Furthermore, we added a small group of healthy Germans from our laboratory (N=13) and compared them with the Punjabi breast cancer patient group (N=13).

1.2 Analysis of Nail Samples

Nail samples from Punjabi test persons were collected by surveyors (PhD candidates) of Punjab Technical University during the period of December 2012 to January 2013. The surveyors took detailed patient information about health issues and lifestyle. Surveyors had been instructed to reject chemically treated hair and painted nails. While we originally had samples for 50 healthy and 50 cancer patients, we had to eliminate nail samples due to insufficient sample weight. For testing, we accept samples weighing between 20 and 60mg.

The mean nail sample weight for the healthy Punjabi group (N=83) was 45.57mg. For the European group (N=83), the mean nail sample weight was 46.6mg. All nail samples were tested at the Micro Trace Minerals Laboratory in Germany. The mean sample weight for the cancer relative group (N=34) was 39.46mg; for the cancer group (N=34) it was 42.49mg. For our healthy German laboratory group (N=13), mean sample weight was 49.86mg, and for the breast cancer group (N=13), the mean sample weight was 40.53.

In the laboratory, samples were soaked with metal-free acetone for 30min, and rinsed three times with hot water and then three more times with ultrapure water. Thereafter, samples were dried in a special drying oven before weighing.

For sample digestion, certified ultrapure nitric acid was used. Digestion took place in a closed-vessel microwave digestion system. Ultrapure water was used for final sample dilution and the elemental analysis was performed via inductively coupled plasma mass spectrometry (ICP-MS) utilizing collision/reaction cell methods coupled with ion-molecule chemistry, a reliable new method for interference reduction.

Through MS-IC analysis, we routinely tested 59 elements for which we evaluated the Limit of Quantitation (Table 3).

1.3 Reference Range Values Used for Nail Sample Data from Punjab

Because nail standards are not available, certified hair standards and in-house standards were used as part of the laboratory quality control and for the validation of results. Reference range values had been developed from apparently healthy people. For the most common metals in blood and urine, governmental agencies provide guidelines. At present, no guidelines are given for hair or nail. Laboratories have to set up their own reference ranges based on the particular populations they serve. Reference intervals serve as the basis of laboratory testing and aid the physician in differentiating between the healthy and diseased patient. Standard methods for determining the reference interval are to define and obtain a healthy population of at least 120 individuals and use nonparametric estimates of the 95% reference interval [34]. The nail reference ranges used in the present study were developed in 1984 by Micro Trace Minerals (MTM), Germany and Trace Minerals International (TMI), USA and are based on a mostly Western population (Europe and US) of about 500 people. In 2013, reference ranges (RR) were re-evaluated (N=1267) and once again in 2014 when we statistically evaluated 544 people. Table 3 shows RRs and pathological ranges as utilized in this study.

1.4 Statistical Analysis

We analysed 59 elements, but our statistical evaluation focused on the 38 standard elements as listed in Table 3, for which reference ranges are available. For all healthy and cancer groups, we evaluated mean value, standard deviation, the number of tests above the 95percentile reference range and the percent of pathological tests. We selected and compared those potentially toxic elements which showed high numbers of pathological values in the Punjab groups and compared them with the European group (Table 1).

We compared the healthy European with the healthy Punjabi group, using Anova (one-way analysis of variance), comparing mean value, variance, P-value and F-value for the elements Al, As, Ba, Cd, Fe, Mn, Ni, Pb, Sr, Ti and U (Table 6).

2. RESULTS

The high concentrations of potentially toxic elements found in the nail samples from people living in the Malwa region largely confirmed the hair metal results of our earlier study. Once again, the breast cancer group (N=13) showed an overall maximum exposure to potentially toxic metals, with the exception of the carcinogenic metals As and Cd. This may support previous studies which had demonstrated higher levels of Cd in breast cancer tissue compared to healthy breast tissues. Metal absorption into cancer tissue may be the cause. Further investigation seems warranted.

The healthy Punjabi group (N=83) showed a considerably higher metal burden than the healthy European group (Table 4, 5, 6). The percentage of pathological values for all the elements shown in Table 1 exceeded the European group. Similarly, the healthy Punjabi group showed significantly higher mean values for Al, As, Ba, Cd, Mn, Ni, Pb, Sr, Ti and U than the European group. The breast cancer group showed the highest mean values for the elements Al, Ba, Ni, Sr, and Ti. High mean values for U were found in all Punjabi test groups (Table 4). When we compared male and females of the various groups, females showed more pathological values for Al. The healthy Punjabi females showed the highest percentage of pathological values for Cd (Table 2).

Table 1. Comparison of percent pathological values in test groups

	# tests	**Al**	**As**	**Ba**	**Cd**	**Mn**	**Ni**	**Pb**	**Sr**	**Ti**	**U**
Punjab -All healthy	83	81	5	63	13	96	23	19	88	51	99
European random 2013+14	83	4	0	2	5	7	7	4	1	2	13
Punjabi cancer relative -healthy	34	94	6	56	9	94	50	29	91	74	100
Punjab all cancer pt	34	85	3	56	0	91	26	15	76	53	100
Punjab Breast cancer pt	13	100	0	69	0	100	39	23	92	85	100
Lab personnel healthy	13	10	0	0	0	0	0	10	0	0	20

 # rounded

Table 2. Comparison of percent pathological values in male and female patients of test groups

	# tests	**Al**	**As**	**Ba**	**Cd**	**Mn**	**Ni**	**Pb**	**Sr**	**Ti**	**U**
Punjab cancer pt- males	13	69	0	54	0	85	**31**	8	62	46	**100**
Punjab cancer pt- females	21	**95**	5	57	0	95	24	**19**	86	**57**	**100**
Punjab healthy males	55	84	4	**65**	7	95	20	26	84	58	98
Punjab healthy females	28	75	**7**	57	**25**	**100**	29	7	**96**	36	**100**
European healthy males	21	0	0	5	5	14	5	0	14	5	24
European healthy females	62	5	0	2	5	5	8	5	8	2	10

 # rounded

Table 3. List of LOQs, reference ranges, and pathological nail ranges

Element tested in nails	Limit of quantitation [mg/kg=mcg/g]	Reference ranges [mg/kg=mcg/g]	Pathological ranges of metals in nails [mg/kg=mcg/g]
Ag	0.020	<1.5	>1.5
Al	0.500	<70	>70
As	0.020	<.87	>.87
B	0.500	2.1	>2.1
Ba	0.020	4	>4
Be	0.020	0.03	>0.03
Bi	0.020	3.14	>3.14
Ca	20.000	550-1850	>1850
Cd	0.001	0.14	>0.14
Ce	0.000	0.26	>0.26
Co	0.001	0.01-.29	>0.29
Cr	0.040	0.1-1.4	>1.4
Cu	0.200	4.45-17.4	>17.4
Fe	2.000	7.0-77	>77
Ga	0.002	0.12	>0.12
Gd	0.002	0.02	>0.02
Ge	0.006	0.28	>0.28
Hg	0.040	0.74	>0.74
I	0.005	0.03-3.7	>3.7
Li	0.002	0.77	>0.77
Mg	10.000	58-197	>197
Mn	0.100	.08-1.45	>1.45
Mo	0.002	0.01-0.15	>0.15
Ni	0.020	5	>5
Pb	0.020	2	>2
Pd	0.100	0.08	>0.08
Pt	0.010	0.02	>0.02
Sb	0.001	1	>1
Se	0.020	0.7-3	>3
Sn	0.002	3.8	>3.8
Sr	0.002	.3-3	>3
Ti	0.019	6	>6
Tl	0.002	0.02	>0.02
U	0.001	0.02	>0.02
V	0.002	0.01-0.21	>0.21
W	0.001	0.03	>0.03
Zn	10.000	80-220	>220
Zr	0.100	2.8	>2.8
# elements	38		

Table 4. Comparing mean values (mcg/g) of test groups

	# tests	Al	As	Ba	Cd	Fe	Mn	Ni	Pb	Sr	Ti	U
Punjabis - healthy	83	250	0.4	8	0.06	301	7	4	6	10	9	0.2
European random	83	22	0.1	1	0.05	23	1	1	1	1	1	0.0
Punjabi healthy- cancer relative	34	363	0.4	7	0.05	444	10	6	12	11	12	0.2
Punjabi all cancer	34	270	0.3	9	0.03	296	7	4	3	10	9	0.2
Punjabi Breast cancer	13	408	0.3	19	0.03	428	10	7	2	14	14	0.2
Lab personnel healthy	13	56	0.1	1	0.02	17	0	1	1	1	0	0.0

Numbers rounded

Table 5. Comparing the total sum of specific metals concentrations in nails

Element	Healthy Punjabis	Healthy Europeans	Factor (rounded)
	N=83	N=83	
Al	20740.71	1800.95	12
As	30.39	6.11	5
Ba	649.28	108.4	6
Cd	5.07	3.76	1
Fe	24997.77	1898.39	13
Mn	622.01	50.46	12
Ni	308.92	123.42	3
Pb	478.19	43.02	11
Sr	793.64	118.67	7
Ti	716.52	95.26	8
U	13.65	1.41	10
	49356.15	4249.84	12

Table 6. Comparing the metal burden of European and Punjabi healthy groups

Summary P-/F-value						
Groups	**Count**	**Sum**	**Mean value**	**Variance**	**P-value**	**Critical F-value**
Al-Eu	83	1800.95	21.7	307.58		
Al-Punj	83	20740.71	249.89	53553.28	7.02E-16	3.90E+00
As-Eu	83	6.11	0.07	0.01		
As-Punj	83	30.39	0.37	0.08	3.54E-16	3.90E+00
Ba-Eu	83	108.4	1.31	10.79		
Ba-Punj	83	649.28	7.82	152.21	6.79E-06	3.90E+00
Cd-Eu	83	3.76	0.05	0.04		
Cd-Punj	83	5.07	0.06	0.01	0.5	3.9
Fe-Eu	83	1898.39	22.87	364.13		
Fe-Punj	83	24997.77	301.18	77955.16	3.76E-16	3.90E+00
Mn-Eu	83	50.46	0.61	0.72		
Mn-Punj	83	622.01	7.49	40.82	5.87E-18	3.90E+00
Ni-Eu	83	123.42	1.49	5.08		
Ni-Punj	83	308.92	3.72	14.5	8.37E-06	3.90E+00
Pb-Eu	83	43.02	0.52	0.23		
Pb-Punj	83	478.19	5.76	911.98	0.12	3.9
Sr-Eu	83	118.67	1.43	2.6		
Sr-Punj	83	793.64	9.56	75.68	2.36E-14	3.90E+00
Ti-Eu	83	95.26	1.15	1.9		
Ti-Punj	83	716.52	8.63	52.36	1.13E-16	3.90E+00
U-Eu	83	1.41	0.02	0		
U-Punj	83	13.65	0.16	0.03	7.59E-12	3.90E+00

3. DISCUSSION

3.1 Nail as an Indicator of Metal Accumulation

Metal intake can be via air, water and food [35,36]. Hair and nail tissue reflect long-term, chronic exposure [37] and the tests' validity has been confirmed. Geographical variations of hair or nail trace element concentrations largely depend on nutritional factors and environmental conditions [38]. People living in geological areas where potentially toxic metals are excessively found, are more prone to chronic metal overexposure. Studies by Ionescu and co-researchers demonstrate that malignant breast tissue have higher metal concentrations than healthy breast tissue [12]. While hair or nail tissue results cannot be directly correlated with breast tissue, all are body tissue, providing an indication of metal exposure and accumulation.

3.2 Aluminium

Darbre states that 'clinical studies showing a disproportionately high incidence of breast cancer in the upper outer quadrant of the breast together with reports of genomic instability in outer quadrants of the breast provide supporting evidence for a role for locally applied cosmetic chemicals in the development of breast cancer. Aluminium is known to have a genotoxic profile, capable of causing both DNA alterations and epigenetic effects, and this would be consistent with a potential role in breast cancer if such effects occurred in breast cells. Aluminium chloride or aluminium chlorhydrate can interfere with the function of oestrogen receptors of MCF7 human breast cancer cells both in terms of ligand binding and in terms of oestrogen-regulated reporter gene expression. This adds aluminium to the increasing list of metals capable of interfering with oestrogen action, termed metalloestrogens' [39]. Mannello et al. found that breast cancer patients had significantly higher levels of aluminium in their nipple aspirate fluids when compared to a control group of healthy women without breast cancer [40], reinforcing earlier findings [41].

The mean value of the total cancer group (N34) was 270/g/Al compared to 250mcg/g/Al for the healthy Punjabi group (N=83), but when we compared the mean (22mcg/g/Al) of the healthy European group (N=83) with the mean of 250mcg/g/Al of the healthy Punjabi group (N=83), the P-value was 7.02E-16, a surprising difference (Table 6).

Water maybe the source of Al. Studies of Punjab water showed Al concentrations above WHO standards of 0.05 mg/L in most of the investigated samples. The average value (0.5231mg/L) for Al was 10times higher [42].

Soil contains Al, as does food. The daily dietary intake of the Mumbai adult population is 6.4 mg Al/day [43] which is equal to the approximate intake of 6mg/day of an Italian population studied [44]. Under normal physiological conditions, the usual daily dietary intake of Al (5-10mg) is almost completely eliminated via renal excretion [45]. Al is also eliminated through faeces.

While several nutritional factors influence the Al uptake, including a healthy nutritional status, the current scientific concern is not the amount of aluminium in food or water, but its bioavailability and the sensitivity of some population groups to aluminium [46].

3.3 Arsenic

Chiou et al. [47] observed a dose-response relationship between the long-term arsenic exposure from drinking artesian well water and the incidence of lung cancer, bladder cancer, and cancers of all sites combined after adjustment for age, sex, and cigarette smoking through Cox's proportional hazards regression analysis. Chen and Ahsan demonstrated that excess arsenic in drinking water at least doubled the lifetime mortality risk from liver, bladder, and lung cancers in Bangladesh [48]. Smith et al assessed evidence indicating that arsenic can cause liver, lung, kidney, and bladder cancer and stated that the population cancer risks due to arsenic in U.S. water supplies may be comparable to those from environmental tobacco smoke and radon in homes [49].

The lowest percentage (0.00%) of pathological results was seen in our healthy laboratory group and the Punjabi breast cancer group. Interestingly, we noted equally high percentages of pathological results (6.02% and 6.0% respectively) in the healthy European group and the group of cancer relatives. In comparison, 4.8% of pathological values were seen in the healthy Punjabi (N=83) compared to 6.02% in the healthy European group (N=83) (Table 1). The mean value of 30.39 mcg/g/As for the healthy Punjabis exceeded the mean value (6.11mcg/g/As) of the European healthy group considerably with a P-value of 3.54E-16 (Table 6).

3.4 Barium

The EPA has determined that barium is not likely to be carcinogenic to humans following ingestion and that there is insufficient information to determine whether it will be carcinogenic to humans following exposure [50], however Barium chromate(VI) is recognized as genotoxic, cytotoxic and carcinogenic [51].

As in our previous hair study, we noted significantly high mean barium values in the nails of all Punjabi groups. The cancer group (N=48) had shown a mean of 64.07mcg/g/Ba and the healthy group (N=50), showed a mean of 30.64 mcg/g/Ba.

Our present study showed significantly higher barium concentrations in the nails of all Punjabi groups with the cancer group (N=34) showing a high mean of 10.44mcg/g/Ba. The mean for the breast cancer group (N=13) was 19.29mcg/g/Ba. As is shown in Table 6, the healthy Punjabis (N=83) showed a considerable Ba burden (p-value 6.79E-06) compared to the European group.

Barium like Ca and Sr belongs to the alkaline earth metals. It has similar general chemical properties with two important differences: the low solubility of Ba sulphate and the large ionic radius, which is similar to that of potassium (K^+). Unlike Pb, Ba does not form heavily soluble sulphides and is not redox-active; it is only found in oxidation number +2 in living organisms and soil.

Barium is used in many industrial applications [52] and may be released into air, soil and water during manufacturing operations [50]. Barium chromate, for instance, is used as a pigment and in the removal of impurities and residual moisture from organic dry-cleaning solvents or from petroleum fuels. Barium sulphate is used in fertilizers, other barium compounds are found in pesticides. Barium manufacturers are located in the Punjab region.

Exposure to Ba can occur in the workplace or from drinking contaminated water. The major sources of barium in drinking water are discharge of drilling wastes, discharge from metal refineries, and erosion of natural deposits. EPA and WHO recommend Ba water levels <2µg/L. Drinking Ba-contaminated water for relatively short periods of time can cause gastrointestinal disturbances and muscle weakness. If consumed for longer periods, kidney damage may result [52]. Since we could not locate data regarding barium testing in Malwa Region water, it is assumed that Ba is not a concern to Punjabi authorities.

3.5 Cadmium

The carcinogenic potential of cadmium might be affected by several factors such as smoking and hormones [53]. Åkesson'sresults support the hypothesis that cadmium may exert oestrogenic effects and thereby increase the risk of hormone-related cancers [54]**.** Garcia-Morales et al suggest that the effects of cadmium are mediated by the oestrogen receptor independent of oestradiol. In addition to its effect on gene expression, cadmium induced the growth of MCF-7 cells 5.6-fold [55].

As in our previous hair study, Table 6 indicates that we only detected a minor cadmium burden in the healthy Punjabi group (0.06mcg/g/Cd), a lesser exposure in the European group (0.05mcg/g/Cd) with an equal mean (0.05mcg/g/Cd) in the Cancer Relative Group (Table 4).

3.6 Iron and Manganese

There is a strong antagonistic interaction between non-heme Fe and Mn at the level of intestinal absorption [56]. Iron (Fe) deficiency will lead to an increase in intestinal absorption of Mn. Fe deficiency is a very common problem in India [57-59].

We found high levels of iron in nails of all Punjabi groups, reflecting malabsorption and iron utilization problems. The highest mean concentration for Fe (444mcg/g) was seen in the healthy cancer relative group, followed by the high mean concentration of 428mcg/g for the breast cancer group (Table 4). The healthy Punjabi group showed a mean value of 301mcg/g/Fe compared to 23mcg/g/Fe for the healthy European group (Table 6).

To further evaluate the Punjabi test group's iron status, we suggest test serum ferritin and transferring levels. Our data suggests an impaired iron utilization problem.

Our previous hair and urine data detected an unusual manganese burden in our test groups of Punjabi children and adults. One again, this present study noted a significant manganese burden in all Punjabi test groups. The most significant difference in manganese exposure was between the healthy Punjabi and the European group, with the Punjabi group's manganese burden exceeding the European group 12fold (Table 5).

High iron and manganese in water and soil can lead to increased bacteria growth. Punjab authorities reported that "the analytical value reveals that our irrigation system has no problem towards Iron. Its concentration falls below the permissible limit of WHO guidelines which is 0.3mg/l" (Directorate of Land Reclamation Punjab 2008).

Once again, we like to indicate that the iron burden of our Punjabi groups may not be due to excessive iron exposure, but due to a disturbed iron metabolism leading to high iron deposits in body tissue. High exposure to manganese and lead, for instance, affect the iron metabolism. A Mongolian study demonstrated that a high accumulation of manganese, iron, lead, cadmium and aluminium leads to oxidative stress, Parkinsonism and arthritis [60].

The nutritional function of manganese is well understood [61,56].In excess, Mn is neurotoxic and affects reproduction [62]. In mammalian cells, Mn causes DNA damage and chromosome aberrations [63]. Floriańczyk et al found that neoplastic cells contained more metallothioneins (by 330%) and more manganese (by 25%) [64]. Spangler and Reid suggest that high Mn levels of groundwater appear to be positively associated with total cancer, colon cancer and lung cancer death rates [65].

Manganese in locally grown foods comes almost entirely from natural sources in the soil and information on soil indicates that the amount of manganese found in soils varies greatly, and may range from 20 to 3,000ppm (mg/kg). Common fertilizers contain manganese sulphate and MnEDTA [66]. Manganese availability increases as soil pH decreases [67,68], however soils in the Malwa region generally have a pH in the alkaline range of 7.5 to 8.0 [69]. In areas where Mn deficiency in soil exists, the deficiency is often (and wrongly) corrected by adding Mn to fertilizers rather than attempting to acidify the soil. Not too long ago, the Soil Science Department of the Punjab Agricultural University (PAU) cautioned farmers about the apparent manganese deficiency in soil and advised the spraying of 0.5% manganese sulphate solution, one kg Manganese sulphate in 200 litre of water per acre.

3.7 Nickel

It has been suggested that Nickel may be essential for human nutrition, but a deficiency state in humans has not been clearly defined, however this metal's health hazard has been established. Nickel dermatitis from contact, respiratory effects and an increased risk of lung and nasal cancer from inhalation exposure have been documented [70-72]. Drinking Ni-contaminated water can cause gastrointestinal distress [73].

Nickel occurs naturally in the environment at low levels and food is the dominant source of nickel exposure in the non-smoking, non-occupationally exposed population. Overall, drinking water appears to contribute only to a minor portion of daily intake, unless nickel levels in groundwater are unusually high [74].

The acceptable range for nickel in drinking water in Europe is 20mcg/L (TrinkwV 2001). The WHO states that based on human data and assuming a 60kg adult drinking 2liters of water per day, the guideline value is 70mcg/l [74].

The mean nickel concentration in the nails of the healthy Punjabi group (3.72mcg/g) exceeded the European group by nearly 3-fold (1.49mcg/g/Ni). The breast cancer group showed the highest mean concentration (6.6mcg/g/Ni) (Table 4, 5, 6).

3.8 Lead

This element is considered mutagenic [75,76]. Steenland et al suggest that, according to animal studies, lead is not genotoxic in vitro. However it is said to promote the mutagenicity of other mutagens, perhaps through inhibition of DNA repair. [A mutagen is also likely to act as a carcinogen: Hutchinson, Dictionary of Science, 1194]. Because elevated lead levels have been found in the nails and hair of our cancer group, a relation between exposure and disease seems probable. When we compared the mean nail concentration of the healthy European group with the healthy Punjabis, an11fold increase was noted in the Punjabi group (Table 5, 6).

While the healthy Punjabi group (N=83) showed a mean concentration of 5.76mcg/g, the healthy Europeans showed a mean level of <1mcg/g/Pb. The cancer relative group (N=34) had the highest mean concentration of 12.21mcg/g/Pb compared to the relatively low mean of 2.71mcg/g/Pb in the cancer group (Table 4). We have no explanation for this.

Pollution is the most common cause of a high Pb burden [77]. A probable source of Pb in Punjab is polluted rainwater, which might come from local and long-distance sources. Lead in drinking water should be less than 0.005mg Pb/L. Unfortunately, clean water is not easily accessible to all Punjabis, and access to uncontaminated water is a pressing issue of great public health importance.

Lead is commonly found near industrial sites and power plants. Lead arsenate pesticides can be another source for Pb in the soil [78,79]. Polluted water used for irrigation may be a potential source of Pb to the soil. A study of water and crops showed that the concentration of Pb, Cd and other metals in sewage-contaminated water was up to 210 times higher than levels in shallow hand pump water [80]. An animal study demonstrated the problem. Shed skins from cobras and wall lizards collected from heavily polluted urban areas contained significantly higher Pb levels than those of the same animals from less polluted rural areas of Punjab [81].

Exposure to Pb produces various deleterious effects on the hematopoietic, renal, reproductive, and central nervous system [82,83], it can induce oxidative stress [84-87],and inhibit the Ca^{++}-ATPase [88] and Na^{+}/K^{+}-ATPase [89]. Inorganic Pb is probably carcinogenic to humans [90]. The US environmental protection agency (EPA) states that “available human evidence is considered to be inadequate to refute or demonstrate any potential carcinogenicity for humans from lead exposure.”

There are two different chemical reasons for the toxic effects of Pb. One is the binding of Pb^{++} to thiol and selenol groups, causing inhibition of various enzymes. Lead, like uranium, can bind to the DNA molecule and catalyse the reaction between DNA and H_2O_2 [91].

3.9 Strontium

Lifetime cancer mortality risk coefficients have been calculated for nearly all radionuclides, including strontium-90. Most of the risk is associated with the high energybeta particle emitted by yttrium-90.

We tested ^{88}Sr, and as in our previous hair study, the mean strontium level of all the Punjabi test groups exceeded the Sr reference range for nails (3mcg/g) (Table 4). When we compared the healthy cancer relatives (N=34) with the total cancer group (N=34), we found a mean value of 10.47mcg/g in the cancer group and a mean of 11.25mcg/g in the healthy Punjab relatives (Table 4). The healthy European group showed a considerably lower body burden of 1.43mcg/g/Sr (Table 6).

^{88}Sr is the most prevalent form, comprising about 83% of natural strontium. The other three stable isotopes and their relative abundance are strontium-84 (0.6%), strontium-86 (9.9%), and strontium-87 (7.0%). We could not locate data regarding the carcinogenicity of ^{88}S.

Stable Sr is found ubiquitously in rocks, soil, dust, and coal and high Sr levels are found naturally in Punjabi water and soil. Governmental tests showed maximum concentrations of up to 1650µg Sr/L (=1.650mg Sr/L) in hand pump water of Malwa region households.

The minimal risk level (MRL) for human oral intake as set by the U.S. Center for Disease Control is 2mg Sr/kg/day, an appreciable amount. An MRL is an estimate of the daily human exposure to a hazardous substance that is likely to be without appreciable risk of adverse non-cancer health effects over a specified duration of exposure. EPA recommends that drinking water levels of stable Sr should not be more than 4mg/L [92].

3.10 Titanium

There is no known biological role for titanium, which is the ninth most abundant element in the Earth’s crust. Ti is not found unbound to other elements in nature. Ti and TiO2 are considered non-toxic. Seawater contains 1ppb Ti, river water about 3ppb. In phytoplankton the Ti concentration is up to 30ppm (mg/kg).

The medical industry has embraced Ti for implants, and the food and cosmetic industry widely uses titanium dioxide in nano-particle form as a colouring additive. While titanium and its compounds are still considered a low health risk, new research indicates that caution is warranted. Otani et al reported case of titanium dioxide inhalation exposure resulting in metal fume fever [93], and since 1997, the US Agency for Toxic Substances and Disease Registry has listed a toxic substance portal for Titanium Tetrachloride. Health effects such as respiratory problems can result from breathing Ti- compound fumes. The EPA has not classified Titanium Tetrachloride as a carcinogen.

While the use of titanium compounds is widespread in Europe, we could not locate information pertaining to the Malwa Region. However, the mean value for the healthy Punjabi group (8.63mcg/g/Ti) exceeded the European group nearly 8fold (mean value 1.15mcg/g/Ti). The breast cancer group showed the highest mean of 13.9mcg/g/Ti compared to the healthy relative group with a mean of 12.1mcg/g/Ti (Table 4, 5, 6).

3.11 Uranium

Our previous study of disabled Punjabi children discovered uranium in the hair and urine of the children and adult test groups (Blaurock-Busch 2010). As a result, uranium became a widely discussed issue in Punjab. The source of the apparent exposure has yet to be determined and speculations created anxieties by linking the U burden to the high rate of cancer.

We tested ^{238}U, the most common isotope of uranium found in nature and as in our previous study on cancer we once again found high uranium concentrations in the nail of Punjabi cancer patients and their healthy relatives. When we compared the healthy Punjabi with the healthy European group, we found a significant difference (p-value 7.59E-12). The mean concentration for the healthy Punjabi test group (0.16mcg/g/U) exceeded the mean concentration of the European group (0.2mcg/g/U) considerably (Table 6). The Punjab cancer group showed a mean of 0.19mcg/g compared with a mean of 0.20mcg/g of the healthy cancer relative group (Table 4).

The Bhabha Atomic Research Centre has tested groundwater samples from four districts of the Malwa region of Punjab (Bhatinda, Mansa, Faridkot and Ferozpur) in collaboration with Guru Nanak Dev University of Amritsar. Of the total samples tested, 42% showed uranium concentration above the Atomic Energy Regulatory Board (AERB) permissible limit of 60µg/L for drinking water [94]. If we consider that the World Health Organization has set the recommended level for uranium in drinking water to <15µg/L [95] and the Federal Environment Agency (Umweltbundesamt) in Germany has lowered the acceptable level for U in drinking water to <10µg/L [96], we noted a considerable difference.

Uranium radiotoxicity or chemical toxicity can be caused by breathing air containing U-rich dust particles, especially in uranium mines and coal mines, or by eating substances containing U or drinking U-containing water. Contaminated water from various areas in the Malwa region has been a source of controversy for years. A study of water samples showed U concentrations ranging from 5.41 to 43.39µg/l [97], which must be considered very high. High U concentration has also been found in soil samples from the Malwa region around Faridkot [98].

The debate about the source of the apparent uranium contamination continues. Some authors speculate that industries like thermal power plants, fertilizer factories, chemical factories, and cement factories are the main source of exposure in Punjab. Others predict that uranium could have originated from the Tosham hills in Haryana state of India where granite rocks with high radioactivity content are found [99]. Geochemical studies suggest that high salinity and TDS influence the mobility of uranium in the groundwater [100].

Uranium is found in higher oxidation numbers in the soil and inside living organisms than Fe, since quadrivalent U is stable under anoxic conditions. Under more oxidizing conditions, even more oxidized U species can be formed, with the hexavalent being most abundant in oxidized soils and other oxidized geological environments, including seawater [101]. Salinity increases the release of natural U.

Phosphate fertilizers are a source of U [102] and the use of phosphate fertilizer, a potential source of metals such as Cd and U, has steadily increased since 2008 [103].Punjab has the highest use of phosphate fertilizers in India. Excessive use of uranium-containing phosphate fertilizers during the summer monsoon season promotes effective leaching of U from topsoil. When agrochemical processes are responsible for mobilizing U that is present in soil, U contamination of ground water can be expected.

Excessive uranium intake and exposure affect human health, however ingested insoluble compounds are poorly absorbed from the gastrointestinal tract and are only retained in the body for a short time, thus they generally are of low toxicity. The main chemical effect associated with exposure to high doses of U and its compounds is renal toxicity [104].

Once in the bloodstream, the U compounds are filtered by the kidneys, where they can cause damage to the kidney cells. Both functional and histological damage to the proximal tubulus has been demonstrated, but little is known about the effects of long-term environmental uranium exposure in humans. Only two small studies with 50–100 study persons have been published on the kidney toxicity of natural uranium from drinking water. They have shown an association of uranium exposure with increased urinary glucose, alkaline phosphatase, and ß-2-microglobulin excretion, as well as increased urinary albumin levels [105]. None of the participants in the present study had listed kidney dysfunction as a health problem.

Uranium mining and lung cancer have been linked [106-111]. Samet and co-workers (1984) demonstrated this association among predominantly non-smoking Navajo men in a population study between 1969 and 1982, but the high rate of breast cancer among Navajo women living on reservation where mining is common has not been officially linked to U. The U intake from food and/or drinking water is unusually high in a human population living in areas where oxidizing soil conditions exist. This is the case in the territories inhabited by Navajo Indians in the United States, in the Iraq and Punjab.

Because uranium is an endocrine-disrupting chemical, populations exposed to uranium may show an increased risk of fertility problems and reproductive cancers. [112]. Busby and colleagues linked cancer and congenital disease to the exposure of depleted uranium as used in warfare Diff [113]. Darolles et al. documented that the genotoxic profile of uranium depends on its isotopic composition. The genotoxicity results from both uranium's chemical and its radiological properties. Enriched uranium has a specific activity 20times higher than depleted uranium. Both affect DNA in ways that can lead to cancer [114].

Nuclear power for civil use is well established in India, and while there are no nuclear power facilities in Punjab, India, there are uranium enrichment facilities in neighbouring Pakistan, two of which are unsafeguarded. Some of India's nuclear power reactors are located in nearby states Rajasthan and Uttar Pradesh. Since we did not test ^{235}U or any of the other U isotopes other than ^{238}U, investigations are needed to clarify issues.

When we compared the percent of pathological values of males and females of the various test groups, both sexes of the Punjabi groups showed near 100% pathological values for U. Interestingly, in the European group, males exceeded females in percent of pathological U-values (Table 2).

4. CONCLUSION

Water, soil, and phosphate fertilizers all seem to play a potential role, causing an increased metal burden in Punjabi people living in the Malwa region. The high concentration of several potentially toxic elements as found in the hair and nails reflect a high toxic metal burden. Toxic metals such as Pb or U lead to oxidative stress and DNA damage [115], thus long-term exposure to multiple toxic elements must be considered a possible cause of the Malwa region's rising cancer rate.

Of concern is the overall high metal burden found in the healthy Punjabi group, which exceeded the European test group in all areas. In Table 5, we compared the total sum of the metal concentration found in nails for those metals outlined in Table 1. These were the metals which were most obviously outside the expected range. The body burden of the healthy Punjabi is more than tenfold that of the European group (Table 5). Females are more burdened by the metals Al and Cd (Table 2).

This unusually high metal burden deserves attention. Adverse health effects of exposure have been reported for many environmental toxins [116,117]. Our data ask for governmental *and* medical preventive measures, including measures to reduce people's contact with environmental toxins and to provide medical aid to reduce the immediate metal burden of Punjabi's people of all ages. Medical tests are available to measure the amount of uranium in urine or stool, although hospitals do not routinely perform these tests. EPA states that these tests are useful for people exposed to uranium. Our research indicates that our Punjabi test population is indeed exposed more than expected. Because most ingested uranium leaves the body in the faeces within a few hours after ingestion, we recommend a large scale study of people living in the Malwa Region. EPA states that urine is a valuable test for the detection of uranium, showing exposure for up to several months after exposure. We do not know of any epidemiological study involving chronic, low grade exposure, but we are of the opinion that such evaluation would benefit all Punjabis. It could determine the seriousness of the exposure and if the link to cancer is indeed valid, and in need of intervention.

Since the overall metal burden of out Punjabi groups is high, we assume that all people living in the Malwa Region show a similar overexposure to potentially toxic metals. Again, an epidemiological study would prove or disprove our hypothesis by evaluating the metal status of more people living in the area. If such a study would support our findings, medical organizations such as the Indian Society for the Study of Metal Toxicology and Chelation Therapy (ISSMTCT) can assist in establishing treatment plans that have the potential of reducing cancer statistics. Early intervention and appropriate detoxification treatments can save money and lives.

While we demonstrated the considerable metal burden of people living in the Malwa Region of Punjab, we have no information about our test groups' individual genetic detoxification ability. Considering that 50 percent of the middle European population is missing the important detoxification enzyme GSTM1 [118] compared to 40.58 percent of North Indian individuals living in the neighbouring state of Haryana who are carriers of the GSTM1 0/0 (null) genotype [119]. At first glance this puts the North Indian population at an advantage, but even a mildly limited detoxification potential coupled with a high toxic exposure as demonstrated by this and previous studies, puts people at risk. Considering the high uranium exposure of unknown sources and Darolles statement that genotoxicity is influenced by isotopic composition (which we can only speculate about at this point), we strongly recommend immediate action. Aside from medical testing, we also recommend chemical and radiological testing of potential sources such as water. Excessive exposure to potentially toxic metals with chemical and radiological properties is considered a cause of cancer birth defects.

No funding was received for this or our previous studies. It was a labour of love for the people of Punjab and elsewhere. To counteract potentially unnecessary anxieties, we recommend to governmental agencies, medical and other groups to fund larger studies that will either confirm or disprove the association as outlined by our data.

CONSENT

All authors declare that written informed consent was obtained from the patient (or other approved parties) for publication of this case report and accompanying images.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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