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The effect of association between inefficient arsenic methylation capacity and demographic characteristics on the risk of skin lesions

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Highlights

- Positive association of arsenic exposure and skin lesions with labour occupations.
- Significant dose response relationship between arsenic exposure and skin lesions.
- Inefficient arsenic methylation capacity significantly associated with skin leisons.
- Variability in 20 houses, 28 persons with and 25 persons without skin problems.

Abstract

This study was conducted in rural Pakistan to assess the dose-response relationship between skin lesions and arsenic exposure and their variation by demographic characteristics. The study included 398 participants (66 participants with skin lesions and 332 without) residing in six previously unstudied villages exposed to ground water arsenic in the range of <1 to 3090 µg L^{-1}. The skin lesions identification process involved interview and physical examinations of participants followed by confirmation by a physician according to UNICEF criteria. Urinary inorganic arsenic (iAs), total arsenic (tAs), monomethylarsonic acid (MMA), and dimethylarsinic acid

(DMA) were analysed to determine methylation capacity, methylation efficiency and the dose-response relationship with skin lesions. Study participants with skin lesions were found to be exposed to arsenic >10 µg L^{-1} with a daily arsenic intake of 3.23±3.75 mg day^{-1} from household ground water sources for an exposure duration of 10-20 years. The participants with skin lesions compared to those without skin lesions showed higher levels of urinary iAs (133.40 ± 242.48 vs. 44.24 ± 86.48 µg g^{-1} Cr), MMA (106.38 ± 135.04 vs. 35.43 ± 39.97 µg g^{-1} Cr), MMA% (15.26 ± 6.31 vs.12.11 ± 4.68) and lower levels of DMA% (66.99 ± 13.59 vs. 73.39 ± 10.44) and secondary methylation index (SMI) (0.81 ± 0.11 vs. 0.86 ± 0.07). Study participants carrying a lower methylation capacity characterized by higher MMA% (OR 5.06, 95% CI: 2.09-12.27), lower DMA% (OR 0.64, 95% CI: 0.33-1.26), primary methylation index (PMI) (OR 0.56, 95% CI: 0.28-1.12) and SMI (OR 0.43, 95% CI: 0.21-0.88) had a significantly higher risk of skin lesions compared to their corresponding references after adjusting for occupation categories. The findings confirmed that inefficient arsenic methylation capacity was significantly associated with increased skin lesion risks and the effect might be modified by labour intensive occupations.

Keywords: Arsenicosis, skin lesions, hyperpigmentation, keratosis, monomethylarsonic acid (MMA), methylation capacity.

1. Introduction

Arsenic (As) exposure from drinking water has placed about 200 million people worldwide at risk of arsenic induced health hazards (National Research Council, 2001). Epidemiological studies have revealed the associations between arsenic exposure and multiple health effects. These include developmental effects, neurotoxicity, diabetes, pulmonary disease and cardiovascular disease (Agency for Toxic Substances and Disease Registry, 2007). Arsenic is a recognized carcinogen causing cancer of the skin, liver, lung, kidney, prostate and bladder (International Agency for Research on Cancer, 2012; Mendez et al., 2017; Hong et al., 2014). Skin lesions are a typical sign of arsenic toxicity appearing after a persistent arsenic ingestion for 5-10 years (Lien et al., 1999; Guha Mazumder et al., 1998). There is considerable evidence of the prevalence of arsenical skin lesions in Bangladesh (Ahsan et al., 2006), India (Guha Mazumder et al., 1998), Mongolia and China (Sun, 2004).

Inorganic arsenic (iAs) ingested from drinking water is metabolized in the human body first by its methylation to monomethylarsonic acid (MMA) and then to dimethylarsinic acid (DMA), resulting in iAs excretion from the body as MMA and DMA (Vahter, 2002). Earlier studies have revealed the relationship between urinary arsenic metabolites and arsenic induced skin disorders (Lindberg et al., 2008; Kile et al., 2011). However, the individuals within the same region or population may have different urinary arsenic levels and methylation capacity even when exposed to the same level of arsenic (Vahter, 1999). This suggests there may be variable disease susceptibility among the exposed persons within a population. Nevertheless, the associations between inadequate arsenic methylation capacity and arsenic-induced health effects may be further influenced by demographic and socio-economic

features, inter-individual variability, genetic or geographical variations (Chen et al., 2013; Lindberg et al., 2010; Steinmaus et al., 2006).

Earlier studies in Pakistan (Fatmi et al., 2013; Fatmi et al., 2009; Ahmed et al., 2014) have assessed the association between water and/or urinary iAs concentrations and the prevalence of skin lesions. This investigation focused on the influence of urinary arsenic metabolites and arsenic methylation capacity on disease susceptibility which is, as yet, unstudied. The prevalence of arsenic related skin manifestations had not been scientifically investigated in this study population and hence evaluated as a biological marker of individual exposure. Moreover, to address the arsenic mitigation challenges, identifying the risk groups in the population of arsenic affected regions is also required (National Research Council, 2001; Jakariya et al., 2005).

2. Methodology

2.1 Study Design and population

The present work is a cross-sectional study involving individuals exposed to arsenic from six villages in the districts of Kasur, Sahiwal, Bahawalpur and Rahim Yar Khan, Pakistan. Our previous study showed that drinking water was the primary source of arsenic exposure beyond the WHO provisional guideline value (10 μg L^{-1}) in the selected villages (Rasheed et al., 2017a). Selection of sample size, recruitment of study participants and demographic characteristics have been published elsewhere (Rasheed et al., 2017b). The 398 non-smoking participants recruited had lived in the study villages for the last 5 years and children (<5 years) by birth and provided consent to being interviewed and physically examined. Health care services in these rural settings were not well organized and no systematic patient records were available to track their arsenic related medical history.

2.2 Physical examination of skin

Initially, study participants were observed and interviewed at their houses by the trained non-physician health workers to record observations on general health status and to specifically screen the individuals with cutaneous signs of skin lesions. Unlike skin cancer, which takes decades to develop, these lesions can appear within a few years of exposure and usually progress through stages. The diagnostic guidelines of the UNICEF clinical diagnostic manual (Sun Guifan et al., 2004) were followed in this screening process. The interviewers, unaware of the health status of the participants, interviewed them using a structured questionnaire that collected information on general wellbeing and visible skin lesions were digitally photographed without facial identification.

Following the steps indicated in Figure-1, initially screened individuals (n=80) were re-examined after a week at the basic health unit (BHU) of each village by a physician with expertise in detection and diagnosis of skin lesions. In accordance with the earlier mentioned diagnostic guidelines (Sun Guifan et al., 2004), hyperpigmentation was symptomized as raindrop-like spots, diffused dark brown spots or darkening of the skin on the limbs or chest, back, and abdomen. Keratosis was identified as thickening of the skin of the palms of hands or the soles of feet, or small flanges (0.4 to 1 cm in diameter) emerged as small corn-like elevations on palms and soles.

Initially screened individuals were physically examined to ascertain the presence, shape and location of visible skin lesions. Out of 80 individuals initially screened as patients, 14 cases were confirmed as not having arsenic induced skin lesions. Thus, the study population was grouped into two subgroups including participants with arsenic specific skin lesions (n=66) and those without such skin lesions (n=332).

2.3 Measurement of Urinary Arsenic Metabolites

The spot urine samples were collected from all participants in a labelled sterile 2 oz polyethylene urine collection container and kept in an ice box for three hours. Exactly 1 mL of urine was kept separately for creatinine (Cr) determination. All urine samples were then immediately transferred to the National Water Quality Laboratory at −20 ℃, where creatinine was determined. All samples were then shipped with dry ice to the Brooks Applied Laboratory (BAL), USA by air and stored at −70 °C, and finally measured for urinary arsenic metabolites within 4 months. Three of the study participants did not provide their urine samples. In total, 395 samples were collected, as well as field duplicates (4% of samples, n=15). Due to spillage during transportation, ten samples did not have enough volume for arsenic speciation. Thus, the Brooks Applied Laboratory (BAL) received 395 samples for total arsenic and 385 samples for arsenic speciation. Urinary creatinine concentration was measured by means of the kinetic Jaffe method using a colorimetric auto-analyzer (Hitachi Ltd., Tokyo, Japan) based on the reaction between creatinine and alkaline picrate (Bonsnes and Taussky, 1945). Concentrations of urinary arsenic species were adjusted using urinary creatinine to correct for variable water excretion rates at the time of specimen collection (Barr et al., 2005). This adjustment was done by dividing the concentration of arsenic metabolites (μg L^{-1}) by U-Cre (g L^{-1}) to express urinary arsenical species as μg g^{-1} creatinine. Frozen urine samples were thawed to room temperature and centrifuged at 3000 rpm for 10 min and the resultant supernatants were used for arsenic analysis. The supernatants were diluted 10-fold with ultrapure water and analyzed. Total arsenic was measured using inductively coupled-plasma dynamic reaction cell-mass spectrometry (ICP-DRC-MS) on a ELAN DRC II ICPMS (Perkin Elmer SCIEX, Concord, Ontario, Canada) following U.S.

Environmental Protection Agency method 1638 mod. (U.S. Environmental Protection Agency, 1996). Urinary arsenic speciation i.e. arsenate (AsV), arsenite (AsIII), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA) and arsenobetaine (AsB) were measured on an anion-exchange high-performance liquid chromatography system (Dionex GP-40) coupled to an inductively coupled plasma – mass spectrometer (ICP-MS) (Agilent 7700x ICPMS, Agilent Technologies) following the proprietary BAL method. Aqueous samples were filtered through a 0.45-µm filter and an aliquot injected onto an anion-exchange column. Measures used to ensure appropriate preservation of MMA and DMA species in urine samples included sample preservation and preparation at low temperatures, immediate freezing upon collection, least sample treatment before analysis, and rapid speciation when analysed. Whilst As(III) can oxidize to As(V) during sample transport, storage, and preparation, these are expressed as total iAs (i.e. As(III)+As(V). The limits of detection were 0.1 µg L^{-1} for tAs, As(III), DMA, and AsB, 0.3 µg L^{-1} for As(V) and 0.2 µg L^{-1} for MMA.

The proportions of urinary arsenic metabolites (iAs%, MMA% and DMA%) and methylation indices, the primary methylation index (PMI) and secondary methylation index (SMI) were calculated to reflect the arsenic methylation capacity. The arsenic methylation indices were defined as the percentages of iAs%, MMA% and DMA%, calculated by dividing the concentration of each species by the sum of iAs, MMA and DMA. The PMI was calculated as the ratio between MMA+DMA and tAs (equation-1), and the SMI as the ratio between DMA and MMA+DMA (equation-2), (Sun et al., 2007).

$$PMI = \frac{MMA + DMA}{tAs} \quad (1)$$

177

$$SMI = \frac{DMA}{(MMA + DMA)} \quad (2)$$

178

179 Quality assurance of urinary arsenic species data was provided by the analysis of
180 NIST (National Institute of Standards and Technology) traceable standard reference
181 materials (SRMs-1640A, trace elements in natural water). Background
182 contamination was monitored using laboratory fortified blanks for urine analysis.
183 Duplicate measurements were made on 10% (n = 40) of urine samples for total
184 arsenic and arsenic species (Table-1). The reliability of the arsenic species
185 determination was evaluated by analysing samples in duplicate and spiking the
186 samples with AsIII, AsV, MMA, DMA and AsB. Data quality in terms of precision,
187 accuracy, method reporting limits (MRLs), method detection limits (MDLs) and
188 completeness met the criteria established in the BAL's quality assurance project plan
189 (QAPP), i.e. relative percent difference (RPD) of <25%, percent recovery of 75 to
190 125% and completeness of 80%. Field duplicates for urine indicated mean
191 percentage differences of ≤10% for tAs, MMA and DMA (Supplementary Information:
192 Table SI-1).

193 **2.4 Individual exposure assessment**

194 All household ground water samples were collected at the houses of study
195 participants from six selected villages during June-September, 2014 after the skin
196 lesions examinations. These were analysed for total arsenic using USEPA method
197 200.8 (U.S. Environmental Protection Agency, 2008) and arsenic species by the
198 Brooks Applied Laboratory using ion chromatography inductively coupled plasma
199 collision reaction cell mass spectrometry (BAL proprietary method). These data were

published previously (Rasheed et al., 2017a). Daily arsenic intake (mg day^{-1}) was calculated by multiplying the household ground water arsenic concentration (µg L^{-1}) by the daily water intake from the household ground water source (L day^{-1}). Thus, exposure in this study was assessed using urinary arsenic metabolites and tAs of household ground water. In order to reduce the potential bias, the participants and health examiners were unaware of the individual arsenic levels of water samples collected from household ground water sources which were analysed after completion of the survey.

2.5 Covariates

In addition to the primary exposure variable we evaluated other covariates suspected to be associated with arsenic exposure. These covariates included socio-demographic factors i.e. age, sex, body weight, exposure duration, daily water intake, villages and occupation, and were derived from the questionnaire based interviews with study participants, published previously in (Rasheed et al., 2017b).

2.6 Statistical Analysis

Since the urinary arsenic metabolites data had a positively skewed distribution, natural logarithmic transformations were used to normalize their distributions and the means as well as the 95% confidence interval (CI). Mean arsenic concentrations in urine and household ground water were calculated for participants with and without skin lesions. The Student t test and Chi-square test was used to assess the differences of exposure variables between participants with and without skin lesions. Urinary arsenic metabolites and methylation indices were stratified into quartiles (0-25%, 25-50%, 50-75% and 75-100%) when estimating the odd ratios (ORs) for

having skin lesions. Variables measured on a continuous scale, including age, body weight, daily arsenic intake and arsenic exposure, were categorized to evaluate risk. Univariate and multivariate logistic regression analyses were used to evaluate the effect of increasing levels of arsenic intake from water, urinary arsenic metabolites and urinary arsenic methylation indices on the risk of skin lesions. The results of logistic analyses were presented as ORs along with their 95% CIs. Only covariates revealed to be significant in the univariate logistic regression and factors of interest were included in the multivariate regression analysis. We used a p value of <0.05 for statistical significance. Microsoft Excel and SPSS 17.0 (IBM, New York, NY, USA) were used for the statistical analysis.

3. Results

3.1 Characteristics of the study population

The baseline characteristics of all participants by status of skin lesions are given in Table 1. The age, body weight, daily water intake, tAs in household water sources and daily water intake were higher among participants with skin lesions than those without skin lesions. Urinary arsenic metabolites such as tAs, iAs, MMA and DMA were higher in participants with skin lesions than those without skin lesions. AsB, excreted as a result of seafood ingestion, was not detected in this study population. Participants with skin lesions also possessed higher means for urinary iAs%, MMA%, lower urinary DMA% and lower PMI and SMI compared with participants without skin lesions (Table 1).

The distribution of cutaneous signs observed in the study participants (Figure 2) varied; hypopigmentation (9.5%), hyperpigmentation (23.8%), hypo and/or

248 hyperpigmentation (6.3%), melanosis (7.9%), whilst keratosis/hyperkeratosis on the
249 palm or sole was the most prevalent cutaneous sign of arsenicism (47.6%).

250

251 **3.2 Association between Urinary Arsenic Methylation Indices and Skin lesions**

252 Table 2 shows the distribution of subgroups with and without skin lesions by sex,
253 age, daily arsenic intake, villages, body weight and occupation. Males were more
254 likely than females to have skin lesions (OR 1.90, 95% CI: 1.05-3.45). Compared
255 with the participants in the youngest age group (≤16 years), the risk of skin lesions
256 increased nearly threefold for participants in the oldest age group >16 years as
257 indicated by an OR of 3.56 (95% CI: 1.25-10.15).

258 There were no skin problems among participants exposed to ground water tAs levels
259 <10 µg L^{-1}. The association between tAs in water and skin lesion (Table 2) showed a
260 significant increasing linear trend from 10-50 µg L^{-1} (OR 1.00: reference) to >50-100
261 µg L^{-1} (OR 23.4, 95% CI: 3.06-178.68) and >100 µg L^{-1} (OR 219, 95% CI: 29.14-
262 1645.70). Consequently, the OR estimates also increased significantly ($p<0.001$)
263 with increasing arsenic intake (0.001-11.773 mg day^{-1}). Risk was significantly higher
264 for the subgroup in the upper quartile of daily arsenic intake (OR 126, 95% CI: 16.89-
265 939.46) suggesting a dose response effect of arsenic exposure from drinking water
266 intake (Table 2).

267 A direct association was found between body weight and skin lesion risk ($p=0.016$),
268 with a threefold increase with increasing body weight >35 kg (OR 3.63, 95% CI
269 1.273-10.35). Based on the socioeconomic situation, intensity of physical and
270 outdoor activities, and occupations of the study participants they were divided into
271 labour intensive (farmers, wives of farmers and service providers like security
272 guards, drivers etc.) and non-labour intensive subgroups (non-working house wives,
273 students, tailors, teachers and un-employed). The labour intensive category

indicated a higher risk of skin lesions (OR 2.83, 95% CI: 1.48-5.39). At village level, a significant increase in the prevalence of skin lesions was found in arsenic affected villages (Table 2), with the highest prevalence of 67.7% skin lesion in Badarpur (OR 20.31, 95% CI: 7.04-58.57), where 95.8% of hand pumps were contaminated with arsenic.

ORs for association of urinary arsenic metabolites with the risk of skin lesions using multiple logistic regression analysis after adjustment for confounding factors, such as age, sex, daily arsenic intake, villages, body weight and occupation, were determined. A higher degree of effect was found when adjusting with occupational categories, as presented in Table 3. After adjustment for occupation, a significantly higher skin lesion risk was found in the third (OR 6.35, 95% CI: 2.08-19.44; p = 0.001) and fourth quartiles (OR 13.07, 95% CI: 4.30-39.68; p = 0.000) of urinary tAs. A significantly increased risk was found for participants in 4^{th} quartiles of urinary iAs (OR 5.61, 95% CI: 2.48-12.70; p = 0.000) Similarly, a significantly increased risk was found in the 4^{th} quartile of MMA (OR 5.83, 95% CI: 2.57-13.24; p = 0.000). The 3^{rd} and 4^{th} quartiles of urinary DMA showed significantly higher ORs for skin lesions (Table 3).

Participants with the highest urinary iAs% (OR 2.65, 95% CI: 1.22-5.75) and MMA% (OR 5.06, 95% CI: 2.09-12.27) showed a significantly highest risk of skin lesions as compared to their reference quartiles (Table 4). Participants in the 2^{nd} quartiles (OR 0.64, 95% CI: 0.33-1.26) of urinary DMA% showed a significantly higher risk of skin lesions as compared to their reference quartiles before and after adjustment for villager's occupations. A significant increased risk of skin lesions was detected in participants in the 2^{nd} quartile of PMI (OR 0.56, 95% CI: 0.28-1.12) and SMI (OR 0.43, 95% CI: 0.21-0.88) both before and after adjustment (Table 4).

4. Discussion

This was the first cross sectional study to evaluate the dose–response relationship between arsenic exposure and skin lesions in rural Pakistan. Epidemiologic outcomes suggest that arsenic induced skin lesions although non-cancerous may convert to be cancerous with prolonged arsenic exposure (Haque et al., 2003; International Agency for Research on Cancer, 2004; National Research Council, 2001). Human methylation capacity plays an important role in determining arsenic induced disease susceptibility. It is therefore important to assess not only the arsenic methylation indices, but also the aggregated effect of these indices with population specific potential modifiers on arsenic-related disease risk. The population in the study villages was found mainly to be exposed to iAs (<1 to 3090 µg L^{-1}) from their household ground water sources. More than 89% of the household hand pumps exceeded the WHO provisional guideline value for arsenic in drinking water (10 µg L^{-1}), whilst 56% were also found to have iAs above Pakistan's water quality standard for arsenic (50 µg L^{-1}) (Rasheed et al., 2017a). The distribution of skin lesions indicated a lowest prevalence (0.7%) at 10-50 µg L^{-1}, 13.8% at 50-100 µg L^{-1} and 60% at >100 µg L^{-1}.Consequently, a higher prevalence of skin lesions was also found for those with higher daily arsenic intake. Past studies have reported the prevalence of skin lesions at iAs concentrations of <10 µg L^{-1} in China (Yang et al., 2017) and Bangladesh (Ahsan et al., 2006; Argos et al., 2011). Despite a very high arsenic exposure level for the current study population, the prevalence rate of skin lesions was found to be lower than the 22% reported in three villages of rural Bangladesh (Ahsan et al., 2000). Similarly, 41.8% was reported in Inner Mongolia for a population with an arsenic exposure level of 2.3-197.3 µg L^{-1} (Guo et al., 2006). Various demographic and life style factors affect arsenic methylation in arsenic-

exposed populations such as age, sex, ethnicity, genetics, socioeconomic status, smoking, alcohol drinking, exposure route and duration, arsenic species, and nutritional inadequacy for essential vitamins, folate, N-acetylcysteine, glutathione, and zinc (Hsueh et al., 2016). The association between skin lesions risk and demographic characteristics was evaluated using univariate logistic regression. Age, sex, daily arsenic intake, village location, body weight and occupation were revealed to be significant factors. A significantly higher prevalence of arsenic induced skin lesions in males (19.7%) than females (11.4%) suggests a higher susceptibility of males to develop skin lesions. These findings are consistent with other studies conducted in Bangladesh and elsewhere (Vahter et al., 1995; Argos et al., 2011; Rahman et al., 2006). The lower prevalence of skin lesions in female participants underscores the better methylation tendency of women than men, possibly linked with biological (hormones, physiology, genetics) and physical or social (sun exposure, water intake and smoking habits) differences between men and women.

Significantly increased skin lesions risk was found among older participants (>16 years) with an OR of 3.56 (95% CI: 1.25-10.152) compared to those ≤16 years. The probable reasons for higher age related susceptibility to arsenic-induced skin lesions include longer exposure duration, higher sun exposure due to the nature of occupation and daily water intake. Also, lower enzymatic and hormonal activity which are involved in arsenic detoxification, and old age related nutritional inadequacy and lower immunity may be the potential factors (Ahsan et al., 2006; Haque et al., 2003; Wei, 1998; Ahsan et al., 2007). Exposure duration to tAs from drinking water by participants with skin lesions varied between 10-20 years (tAs >100 µg L^{-1}), 14-20 years (As 50-100 µg L^{-1}) and 20 years for (As 10-50 µg L^{-1}) on the basis of consumption duration for household ground water. This suggests that the affected

349 populations would be consuming untreated ground water for several years. Ground
350 water tAs being the direct exposure variable seems to indicate the clear dose related
351 trend for skin lesions risk above >10 µg L^{-1}. This is indicated by 20% increased risk
352 of skin lesions for those exposed to 50–100 µg L^{-1} iAs compared to those with <10
353 µg L^{-1}, and this risk further increased more than 9.5-fold (OR 219, 29.14-1645.7) for
354 the exposure >100 µg L^{-1} (Table 2).

355 The study showed that male, older, and/or heavier participants were more likely to
356 be at risk of arsenic exposure (Table 2). An increased risk of skin lesions (OR 2.83,
357 95% CI: 1.48-5.39) was found among participants involved in labour intensive
358 (farmers, wives of farmers and service providers like security guards, drivers etc.)
359 occupations compared to the non-labour intensive (non-working house wives,
360 students, tailors, teachers and un-employed) occupations (Table 2). Occupationally,
361 the majority of the study participants were farmers (n=186) working outdoors and
362 generally had sun exposure for 8-10 hours per day. The labour intensive occupations
363 also included wives of farmers (n=56) contributing in the crop fields with their farmer
364 husbands, possibly having higher sun exposure resulting in higher drinking water
365 intake. The labour intensive occupations may also be associated with other risk
366 enhancing factors such as low socio-economic status and poverty related
367 malnutrition.

368 Simultaneous adjustment of significant confounding factors (Table 2) in multivariate
369 regression analysis has showed an overall model significance for villager's
370 occupation and thus adjustments were made for labour intensive and non-labour
371 intensive occupation categories. This model adjustment was utilized to show that the
372 association between skin lesions and urinary arsenic metabolites (tAs, iAs, MMA,
373 DMA), methylation capacity (iAs%, MMA%, DMA%) and methylation efficiency (PMI

374 and SMI) might be enhanced by intensive physical activities and higher sun
375 exposure.

376 The influence of occupation is obvious from the decrease in adjusted ORs than
377 unadjusted ORs for methylation capacity and efficiency indicators. Contrary to the
378 studies by Haque et al. (2003) indicating ORs of 3.1 (51-99 μg L^{-1}), and 5.0 (>150 μg
379 L^{-1}), and (Guo et al., 2006) showing ORs of 15.50 (51-99 μg L^{-1}), and 25.70 (>150
380 μg L^{-1}), this study showed much higher ORs of arsenical skin lesions for increasing
381 arsenic exposure from household water sources. The impact of metabolically
382 produced arsenic on the significantly increased skin lesions risk was obvious among
383 the skin lesions subgroup in the 4th quartiles of urinary tAs, iAs, MMA, DMA, iAs%
384 and MMA%, 2nd quartiles of DMA%, PMI and SMI.
385 A significantly increasing trend was found with increasing levels of urinary tAs (>247
386 μg g^{-1}) indicated by a 2.4-fold increased odds of skin lesions (Table 3). Compared to
387 this, Argos et al. (2011) reported 2.4-fold increased odds of skin lesions at a
388 comparatively higher level of urinary tAs (i.e. >393 μg g^{-1}). Intermediary by-products
389 of iAs such as MMA and DMA are methylated via similar metabolic pathways,
390 however MMA is considered more toxic than iAs and DMA (Chen et al. (2013). The
391 trivalent forms of MMA produced in this process were considered to be more toxic
392 than pentavalent MMA (Hirano et al., 2003; Petrick et al., 2001). The limited
393 evidence on the health risk potential of ingested arsenic compared to metabolically
394 produced MMA or DMA has given impetus to assess the relationship between
395 arsenic related health effects and methylation capacity. Following this, the study
396 results showed the association of daily arsenic intake with skin lesions incidence in a
397 dose-dependent manner for absolute concentrations of urinary arsenic metabolites
398 (Table 3). Increasing ORs from lower to upper quartiles of urinary arsenic

399 metabolites demonstrated that the magnitude of exposure is directly related to the
400 presence of skin lesions. Sub-groups with skin lesions indicated significantly higher
401 mean values of urinary iAs%, MMA%, lower DMA%, PMI and SMI compared to
402 those without skin lesions (Table 1). These findings are also in close agreement with
403 the studies by Steinmaus et al. (2006) and Kile et al. (2011), revealing higher levels
404 of urinary MMA% related with the higher risk of lung cancer and skin lesions
405 respectively.

406 Arsenic methylation mechanisms are still controversial, however the ORs for arsenic
407 induced diseases have been found higher in those with higher MMA% (Chen et al.,
408 2013; Zhang et al., 2014; Li et al., 2015). Of all the methylation indices determined in
409 this study, MMA% in upper quartiles (OR 5.06, 95% CI: 2.09-12.27) indicated the
410 highest skin lesions risk compared to its corresponding reference (OR 1.00).
411 Comparing the current study findings with earlier studies, MMA% is suggested to be
412 an underlying reason of higher dermatoxicity and also a potential biomarker for
413 preliminary screening of individuals suspected to be at an arsenic induced health
414 risk.

415 The significantly decreased risk of skin lesions in the fourth quartiles of DMA% (OR
416 0.22, 95% CI: 0.10-0.50) and SMI (OR 0.17, 95% CI: 0.07-0.40) was also in
417 agreement with earlier studies on arsenic induced development delays (Hsieh et al.,
418 2014) and skin lesions (Li et al., 2011)**.** The higher iAs%, MMA% and lower DMA%
419 among the participants with skin lesions depicted inefficient methylation capacity
420 compared to those without skin lesions. This association between inadequate
421 methylation capacity and arsenic induced health effects was found to be consistent
422 with studies on arsenic induced cardiovascular diseases (Chen et al., 2013; Li et al.,
423 2015) and bladder cancer (Chen et al., 2003).

Participants with oral arsenic exposure >50 µg L^{-1} and also having skin lesions showed significant increased (p=0.004) urinary MMA concentration compared to those exposed to tAs through drinking water but without skin lesions. The study participants identified with skin lesions belonged to 47 households. Out of these 47, 20 houses comprising 53 study participants revealed 28 persons with skin lesions, while 25 persons from the same houses showed no skin problems, despite being exposed to the same level of arsenic from their household water sources (Figure 3). Persons within the same house with higher arsenic concentration but with no skin lesions were found to be younger in age than their family members having skin lesions. The fact that some study participants did not develop skin lesions despite similar exposure to arsenic as those who did suggests the possible influence of inter-individual variability and various demographic, biological, genetic and nutritional factors on methylation efficiency. Valenzuela et al. (2009) found that genetic polymorphisms for arsenic (+3 oxidation state) methyltransferase (AS3MT) influence the susceptibility of humans to arsenical skin lesions and these people might be at higher risk for other arsenic induced adverse health effects. Deficiency of nutrients such as proteins, folate, vitamin B_{12} and vitamin B_6 have been emphasized to interfere in arsenic metabolism and toxicity resulting in increased susceptibility to arsenic induced disease e.g. age-adjusted prevalence keratosis (Zablotska et al., 2008). This is indicated by positive correlation between urinary DMA and plasma folate in Bangladesh (Gamble et al., 2005) and negative correlation between the prevalence of arsenic-induced skin lesions and proteins intake (Mitra et al., 2004). Nutritional inadequacy may also be the reason for age related susceptibility to skin lesions, especially in case of older participants. The individuals with or without skin

lesions might have suffered from other arsenic related health hazards which need to be further investigated.

The study findings may prove useful in understanding arsenic induced susceptibility to skin lesions, for early detection of skin lesions in communities residing in arsenic-affected regions, and may also be helpful for policy and decision makers. In addition to speciation for MMA, future studies should also evaluate the impact of association between arsenic methylation capacity and other modifiable risk factors on the variations in arsenic induced health hazards.

5. Conclusions

The occupation adjusted odd ratios suggested a significant dose response relationship between various exposure levels measured, using either water or urinary total arsenic, and the risk of skin lesions. The study supports the findings of other cross sectional studies demonstrating the inefficient methylation capacity in association with higher iAs% and MMA%, lower DMA%, PMI and SMI among individuals affected with arsenic induced diseases. The significantly increased risk of MMA% in older individuals with skin lesions indicates the metabolic barriers to converting MMA to DMA, also underscoring the probability of other arsenic induced health hazards among the exposed population. Even though skin lesions occur at exposure to 10-50 µg L^{-1} arsenic, countries including Pakistan currently follow a drinking water standard for arsenic of 50 µg L^{-1}. This may place many people at risk of developing arsenic induced adverse health effects with persistent exposure. Our findings support an association between skin lesions and a higher intake of arsenic concentrations beyond the WHO provisional guideline value for arsenic in drinking water (10 µg L^{-1}).

6. Ethical Approval

The study was approved by the National Bioethics Committee of Pakistan and University of Leeds Research Ethics Committee.

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672 **Table 1: The baseline characteristics of the study participants**

Characteristics	n	Overall (Mean±SD)	with skin lesions (Mean±SD) (n=66)	Without skin lesions (Mean±SD) (n=332)[a]	p-value
Age of participants (years)	398	35.74±16.99	39.92±15.19	34.91±17.23	0.001***
Body weight (kg)	398	56.66±19.92	64.45±15.43	55.11±20.37	0.0005***
Daily total water intake (L person^{-1} day^{-1})	398	3.47±0.955	3.98±0.97	3.38±0.92	0.0005***
Daily arsenic intake from water (mg day^{-1})	398	0.78±2.01	3.23±3.57	0.32±0.98	0.0005***
tAs conc. in household water sources (µg L^{-1})					
Chak-46/12-L	121	62.28±39.42	113.38 ± 47.82	53.34 ± 30.09	-
Chak-48/12-I	54	275.30±335.97	497.51 ± 433.07	164.17 ± 204.30	-
Chak 49/12-I	75	54.57±26.18	81.99 ± 13.37	51.75 ± 25.58	-
Basti Balochan	44	24.88±0.68	NA	24.88 ± 6.81	-
Badarpur	34	1605.64±882.51	1874.26 ± 776.88	1043.98 ± 854.0	-
Basti Kotla Arab	70	14.784±13.96	NA	14.784 ± 13.95	-
Overall tAs	398	209.08±519.20	828.46±934.28	85.96±245.38	0.0005***
Urinary arsenic concentration (µg g^{-1} Cr)					
Urinary tAs	395	407.66±659.34	760.48±883.81	336.87±580.81	0.0005***
iAs	395	59.52±131.45	133.40±242.48	44.24±86.48	0.0005***
MMA	385	47.59±71.60	106.38±135.04	35.43±39.97	0.0005***
DMA	385	255.19±301.20	464.70±518.34	211.85±208.90	0.008**
Urinary arsenic proportions and methylation indices					
iAs%	395	15.05±8.99	17.75±9.66	14.50±8.77	0.001***
MMA%	385	12.65±5.13	15.26±6.31	12.11±4.68	0.0005***
DMA%	385	72.29±11.28	66.99±13.59	73.39±10.44	0.006**
PMI	385	0.85±0.09	0.82±0.10	0.86±0.09	0.032*
SMI	385	0.85±0.08	0.81±0.11	0.86±0.07	0.003**

673 [a]n varies for results of urinary arsenic metabolites and methylation indices.
674 SD: Standard deviation
675 *p ≤ 0.05* , p ≤ 0.01**, p ≤ 0.001****
676

Table 2. The ORs for skin lesions by levels of demographic and lifestyle factors

Co-variates	Total number of participants (n=398)	Without skin lesion (n=332)	With skin lesion (n=66)	Prevalence %	p-value	OR (95% CI)
	n	n	n			
Sex						
female	149	132	17	11.4	0.034*	1.00 (ref)
male	249	200	49	19.7		1.90 (1.05, 3.45)
Age						
≤16 years	66	62	4	6	0.018*	1.00 (ref)
>16 years	332	270	62	18.67		3.56 (1.25,10.152)
tAs in household water sources (µg L^{-1})						
10-50	147	146	1	0.68	p<0.001***	1.00 (ref)
50-100	123	106	17	13.82		23.4 (3.06,178.68)
>100	80	32	48	60		219.0 (29.142,1645.7)
Daily arsenic intake (mg day^{-1})						
Q1:0.001-0.070	99	99	0	0	p<0.001***	-
Q2:0.071-0.160	100	99	1	1		1.00 (ref)
Q3:0.162-0.330	99	90	9	9.1		10.01 (1.24,80.59)
Q4:0.332-11.773	100	44	56	56		126.0 (16.89,939.46)
Villages						
Chak 49/12-I	75	68	7	9.3	p<0.001***	1.00 (ref)
Chak-46/12-L	121	107	18	14.9		1.70 (0.67, 4.28)
Chak-48/12-I	54	34	18	33.3		4.86 (1.86, 12.71)
Badarpur	34	12	23	67.7		20.31 (7.04, 58.57)
Basti Balochan	44	44	0	0		0
Basti Kotla Arab	70	70	0	0		0
Body weight (kg)						
≤ 35 kg	67	63	4	6	0.016*	1.00 (ref)
> 35 kg	331	269	62	18.7		3.63 (1.273,10.35)
Occupation						
Labour non-Intensive	149	136	13	8.7	0.002**	1.00 (ref)
Labour intensive	249	196	53	21.3		2.83 (1.48,5.39)

CI, Confidence interval
Q: Quartile
$p \leq 0.05$, $p \leq 0.01$**, $p \leq 0.001$****

Table 3. The logistic regression analysis of ORs, unadjusted and adjusted[a], for skin lesions risk by level of urinary arsenic metabolites

Urinary arsenic exposure measures (quartiles)		With skin lesions (n=66)	Without skin lesion (n=332)	Unadjusted OR (95% CI)	p-Value	Adjusted OR[a] (95% CI)	p-value
Urinary tAs (µg g^{-1} Cr)[b]	7.78-123.42	4	94	**1.00 (ref)**	-	**1.00 (ref)**	*p ≤ 0.001****
	123.58-246.94	11	88	2.94 (0.90-9.57)	0.074	3.14 (0.96-10.31)	0.059
	247.19-426.67	21	78	6.33 (2.08-19.21)	0.001***	6.35 (2.08-19.44)	0.001**
	441.12-8743.59	30	72	9.79 (3.30-29.05)	0.0005***	13.07 (4.30-39.68)	*p ≤ 0.001****
Urinary iAs (µg g^{-1} Cr)[b]	0.14-13.796	9	87	**1.00 (ref)**		**1.00 (ref)**	*p* ≤ 0.001***
	13.81-28.58	8	88	0.88 (0.32-2.38)	0.8	1.00 (0.37-2.75)	0.993
	28.66-56.58	14	82	1.65 (0.68-4.02)	0.27	1.81 (0.74-4.47)	0.195
	58.24-1411.11	35	75	4.51(2.04-9.99)	0.0005***	5.61 (2.48-12.70)	*p ≤ 0.001****
Urinary MMA (µg g^{-1} Cr)[c]	0.08-10.89	9	87	**1.00 (ref)**		**1.00 (ref)**	*p ≤ 0.001****
	10.9-27.03	6	90	0.64 (0.22-1.89)	0.423	0.76 (0.26-2.24)	0.617
	27.32-54.44	16	80	1.93 (0.81-4.62)	0.138	2.09 (0.87-5.05)	0.101
	54.49-615.31	35	75	4.51 (2.04-9.99)	0.0005***	5.83 (2.57-13.24)	*p* ≤ 0.001***
Urinary DMA (µg g^{-1} Cr)[c]	0.077-90.90	8	88	**1.00 (ref)**		**1.00 (ref)**	*p ≤ 0.001****
	91.48-164.94	10	86	1.28 (0.48-3.39)	0.621	1.38 (0.52-3.70)	0.520
	165.42-302.10	19	77	2.71 (1.12-6.55)	0.026*	2.78 (1.14-6.77)	0.024*
	307.80-2353.5	29	81	3.94 (1.70-9.11)	0.001***	4.93 (2.08-11.64)	*p ≤ 0.001****

CI, confidence interval
Cut off points were determined by quartiles of urinary arsenic metabolites of overall study participants.
p ≤ 0.05, p ≤ 0.01**, p ≤ 0.001****
[a] ORs were adjusted by participant's occupation
[b] n=395,
[c] n=385

Table 4. The logistic regression analysis of the ORs unadjusted and adjusted[a], for skin lesions risk in relation to urinary arsenic methylation indices

Urinary arsenic exposure measures (quartiles)		With skin lesions (n=66)	Without skin lesion (n=329)	Unadjusted OR (95% CI)	p-value	Adjusted OR[a] (95% CI)	p-value
%iAs	2.47-10.08	11	85	**1.00 (ref)**		**1.00 (ref)**	0.012*
	10.14-12.98	9	87	0.80 (0.32-2.03)	0.637	0.80 (0.31-2.06)	0.648
	12.99-17.0	19	77	1.91 (0.85-4.26)	0.116	1.79 (0.79-4.05)	0.160
	17.01-75.28	27	83	2.51 (1.17-5.39)	0.018*	2.65 (1.22-5.75)	0.014*
MMA%[b]	0.63-8.97	7	89	**1.00 (ref)**		**1.00 (ref)**	0.002**
	9.01-11.98	13	83	1.99 (0.76-5.23)	0.162	2.20 (0.83-5.84)	0.113
	11.98-15.90	16	80	2.54 (1.00-6.50)	0.051	2.72 (1.05-7.01)	0.039*
	15.92-42.62	30	80	4.77 (1.98-11.45)	0.0005***	5.06 (2.09-12.27)	*p ≤ 0.001***
DMA% [b]	8.5-68.51	28	69	**1.00 (ref)**		**1.00 (ref)**	*p ≤ 0.001***
	68.57-73.93	20	75	0.66 (0.34-1.27)	0.213	0.64 (0.33-1.26)	0.201
	73.98-79.02	9	87	0.26 (0.11-0.58)	0.001***	0.25 (0.11-0.56)	0.001**
	79.08,91.57	9	101	0.22 (0.10-0.49)	0.0005***	0.22 (0.10-0.50)	*p ≤ 0.001***
PMI [b]	0.247-0.829	27	69	**1.00 (ref)**		**1.00 (ref)**	0.001***
	0.830-0.870	19	78	0.62 (0.32-1.22)	0.166	0.56 (0.28-1.12)	0.099
	0.870-0.899	9	87	0.26 (0.12-0.60)	0.001***	0.25 (0.11-0.58)	0.001***
	0.899-0.975	11	98	0.29 (0.13-0.62)	0.001***	0.28 (0.13-0.60)	0.001***
SMI [b]	0.293-0.814	30	67	**1.00 (ref)**		**1.00 (ref)**	*p ≤ 0.001***
	0.814-0.856	15	80	0.42 (0.21-0.84)	0.015*	0.43 (0.21-0.88)	0.020*
	0.856-0.894	13	83	0.35 (0.17-0.72)	0.005**	0.34 (0.16-0.71)	0.004**
	0.895-0.976	8	102	0.18 (0.08-0.41)	0.0005***	0.17 (0.07-0.40)	*p ≤ 0.001***

CI, confidence interval
Cut off points of urinary were determined by quartiles of overall study participants.
p ≤ 0.05, p ≤ 0.01**, p ≤ 0.001****
[a] Adjusted by villager's occupation
[b] n=385

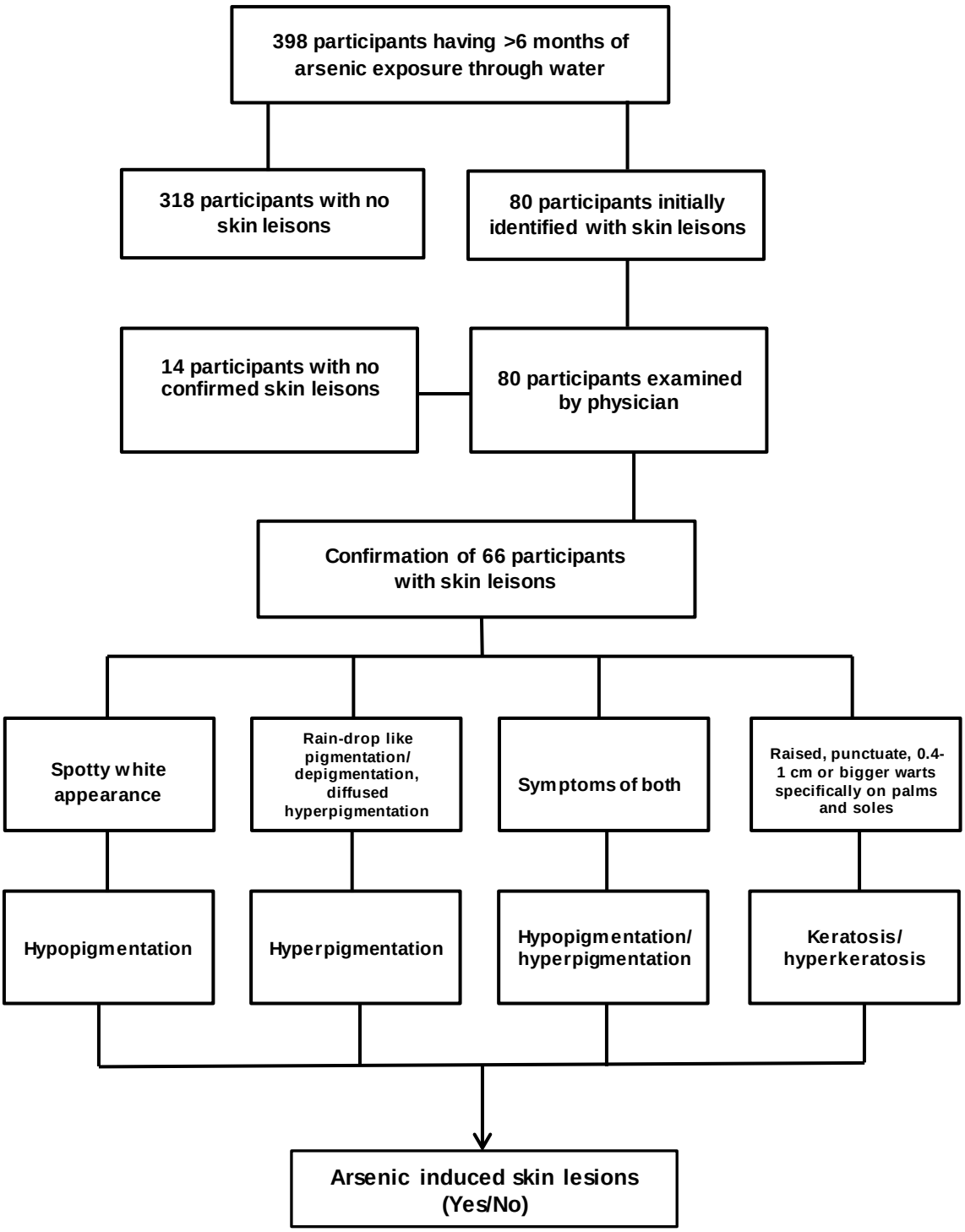
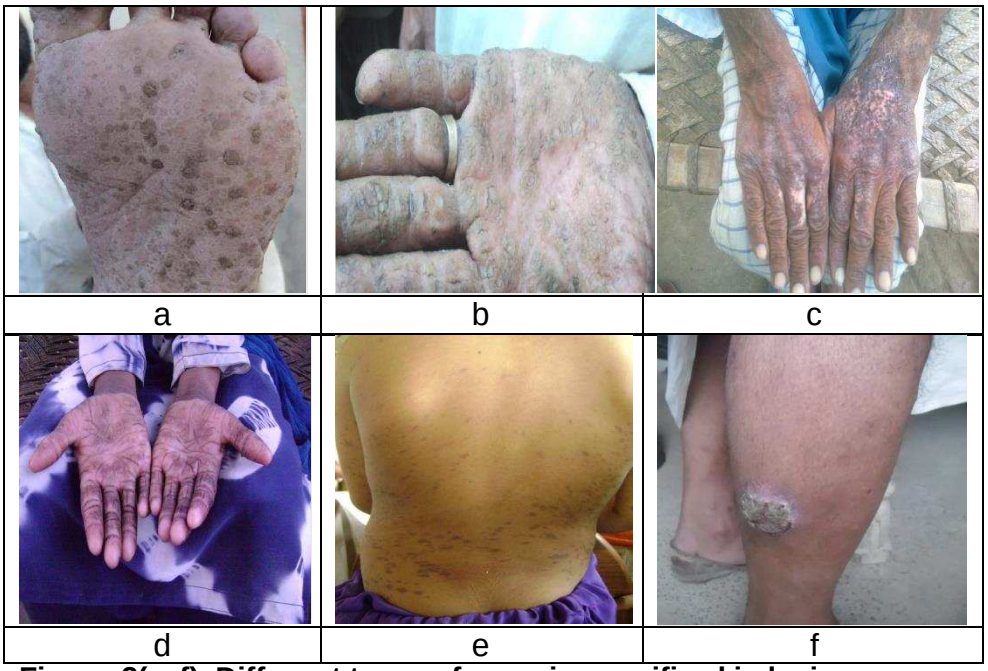


Figure-1: Steps involved in screening of participants with arsenic-induced skin leisons

739



740 **Figure-2(a–f): Different types of arsenic-specific skin lesions**
741 (a) Keratosis on sole (b) Keratosis on palm (c) Hypopigmentation on hand (d) Hyperpigmentation on palms (e)
742 Melanosis on trunk (f) Hyperkeratosis on lower limb.
743

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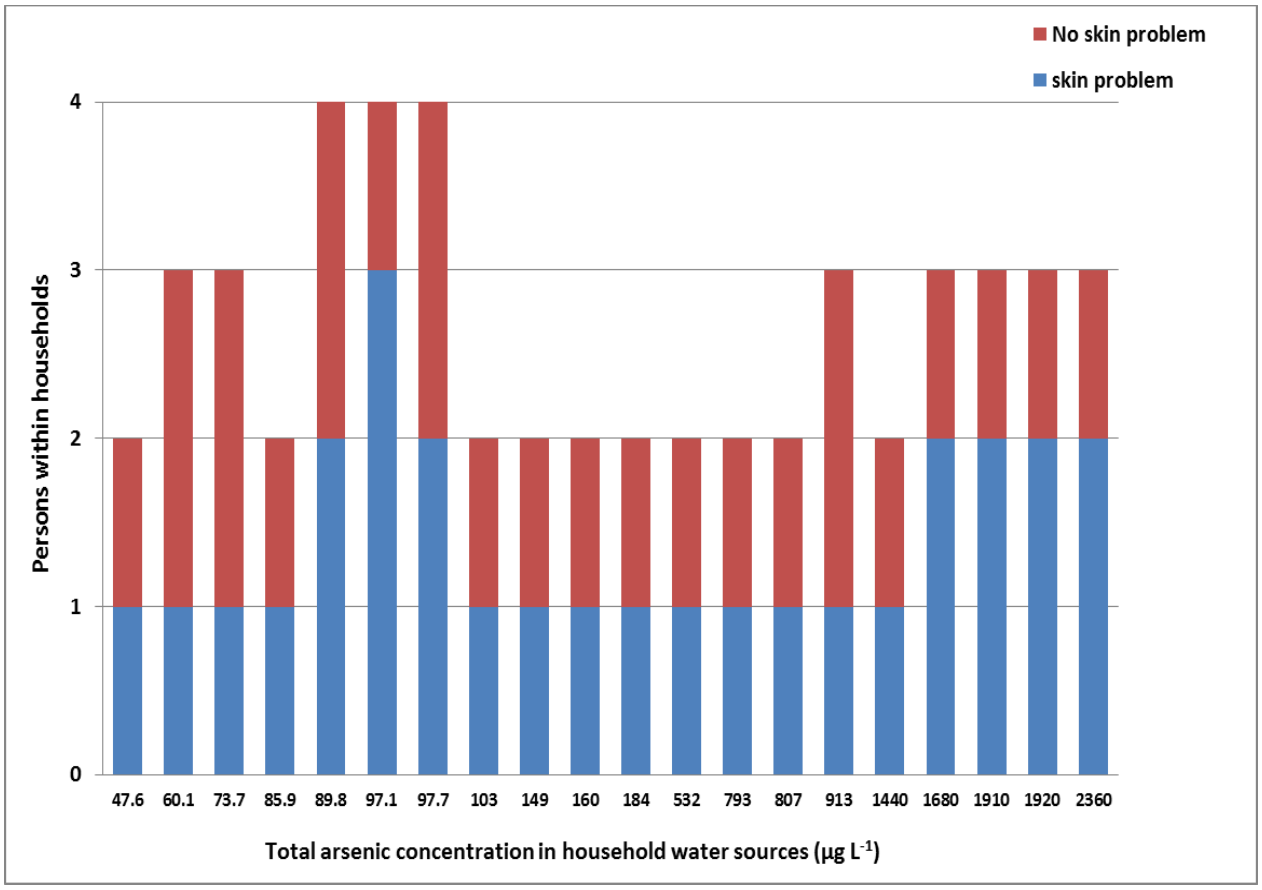

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746 **Figure-3: Households showing tAs concentration in ground water sources and inter-individual**
747 **variability for arsenic induced skin lesions**

748