Lead poisoning in children from townships in the vicinity of a lead-zinc mine in Kabwe, Zambia

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Extensive childhood Pb poisoning in Zambia’s Kabwe mining town may have serious health effects on the children ranging from neurological deficits to deaths.
Abstract

Childhood lead poisoning is a serious public health concern worldwide. Blood lead levels exceeding 5 µg/dL are considered elevated. In Kabwe, the capital of Zambia’s Central Province, extensive Pb contamination of township soils in the vicinity of a Pb-Zn mine and posing serious health risk to children has been reported. We investigated BLLs in children under the age of 7 years in townships around the mine; where blood samples were collected and analysed using an ICP-MS. Almost all of the sampled children had BLLs exceeding 10 µg/dL. Children in these areas could be at serious risk of Pb toxicity as 18% of the sampled children in Chowa, 57% (Kasanda) and 25% (Makululu) had BLLs exceeding 65 µg/dL. Eight children had BLLs exceeding 150 µg/dL with the maximum being 427.8 µg/dL. We recommend that medical intervention be commenced in the children with BLL exceeding 45 µg/dL.

Keywords: Children; lead poisoning; Pb-Zn mine; Kabwe, Zambia.
1. Introduction

Childhood lead (Pb) poisoning is a serious public health concern worldwide (Tong et al. 2000). Exposure to Pb affects multiple organ systems resulting in numerous morphological, biochemical and physiological changes that include hematological disorders, nervous system disturbances and impairment of liver and kidney functions (Lockitch 1993; Al-Saleh 1994; Canfield et al. 2003; Needleman 2004). Young children are particularly vulnerable to Pb exposure and poisoning. This is because young children frequently explore their environment via hand-to-mouth and object-to-mouth activities; behaviors that are likely to increase Pb intake in children from polluted environments such as house dust or yard soils (Calabrese et al. 1997; Manton et al. 2000). Biological factors also play a significant role in increased Pb uptake in children as the average fractional gastrointestinal absorption of Pb is much greater in infants and young children than in adults (Ziegler et al. 1978). Moreover, Pb absorption is increased in the presence of nutritional deficiencies such as iron and calcium, which are more common in children than in adults (Bradman et al. 2001). Children are also more vulnerable to Pb poisoning compared to adults as the central nervous system is most sensitive to Pb toxicity during developmental stages (Bellinger 2004; Lidsky and Schneider 2003). Although the effects of Pb on the nervous system in adults tend to reverse after cessation of exposure (Baker et al. 1985), effects in children tend to persist (Needleman et al. 1990).

Lead concentration in whole blood (BLL) is the main biomarker used to monitor exposure and has been widely used in epidemiological studies (CDC 2009). The Centers for Disease Control and Prevention (CDC 2012) recently revised the blood lead “level of concern” from 10 to 5 μg/dL in response to reports that BLLs < 10 μg/dL can cause neurological abnormalities such as decreased intelligence quotient (IQ) in children.
(Canfield et al. 2003). Therefore, a threshold below which Pb does not result in neurological deficits has not been determined (Needleman 2004). However, individuals differ widely in the BLL at which signs of Pb toxicity appear, with some individuals seeming well at a BLL that in others results in encephalopathy or even death (Bellinger 2004). The detrimental effects of elevated BLLs in the range of 10 to 45 µg/dL are usually subclinical and may include neurodevelopmental impairment (CDC 2002). Generally, BLLs > 10 µg/dL in children are considered elevated and it has been recommended that chelation therapy be initiated at levels ≥ 45 µg/dL (CDC 2002; Needleman 2004). At higher BLLs > 60 µg/dL, clinical symptoms such as abdominal pain and arthralgia become visible in children (Needleman 2004). Moreover, it has been reported that high BLLs exceeding 100 µg/dL can cause encephalopathy, convulsions, coma and death, especially in children (CDC 2002; TNO 2001).

In the last decade, BLLs in children have reduced significantly in a number of developed countries following the phasing out of leaded gasoline (Wilhelm et al. 2006). However, childhood Pb toxicity continues to be a major public health problem in most developing countries. In Africa, major sources of childhood Pb poisoning include Pb mining and smelting, paint and battery recycling (Nriagu et al. 1996; Mathee et al. 2007). The recent Pb poisoning disaster in Nigeria, where more than 400 children died leaving numerous others with long-term neurological impairment including blindness and deafness, was attributed to gold ore–mining and processing, especially that metals were processed in their dwellings (Blacksmith Institute 2011, Dooyema et al. 2012; Lo et al. 2012). In Kabwe Town, the capital of Zambia’s Central Province, extensive Pb contamination of township soils in the vicinity of a Pb-Zn mine has been reported and poses a serious health risk to children in these townships (Tembo et al. 2006; Nakayama et al. 2011). In an earlier study,
Pb poisoning and cases of encephalopathy were recorded in children from a township in the vicinity of the Pb-Zn mine in Kabwe (Clark APL, unpublished data).

Despite extensive Pb pollution in Kabwe, comprehensive studies of Pb exposure and poisoning in children in the vicinity of the mine are rare. In animal studies however, high concentrations of Pb were reported in wild rats (Nakayama et al. 2011; Nakayama et al. 2013) as well as blood and edible organs of cattle (Yabe et al. 2011; Ikenaka et al. 2012) and chickens (Yabe et al. 2013) reared in the vicinity of the mine in Kabwe. Therefore, the objectives of the current study were to investigate BLLs in children under the age of 7 years in townships around the Pb-Zn mine in Kabwe and to identify children with BLLs that require medical intervention.

2. Materials and methods

2.1 Sampling sites

Kabwe town, the provincial capital of Zambia’s Central Province, is located at about 28°26′E and 14°27′S. Kabwe has a long history of Pb-Zn mining. The mine operated almost continuously from 1902 to 1994 without addressing the potential risks of metal pollution. Dense fumes rich in Pb and other metals were emitted from smelters and they polluted the environment in the surrounding communities extensively (Tembo et al. 2006). Despite closure of the mine, scavenging of metal scraps from the abandoned tailings and wastes stored on the mine has continued to serve as a source of metal pollution, especially dusts emanating from the mine dumps.

In the current study, blood samples were collected from children at health centers located in Chowa, Kasanda and Makukulu townships, in May-June of 2012. Kasanda Township lies west to the mine and its center is about 2.2 km from the smelter (Fig. 1).
However, some households in Kasanda are within 1 kilometer of the mine. Makululu Township is a large squatter compound that lies adjacent and to the west of Kasanda Township. These two townships are affected by dust emanating from the mine as the prevailing winds most of the time blow from the east to the west. Most houses in Makululu are made of mud brick walls, mud floors and thatched roofs. Moreover, lots of dust is emitted by vehicles as roads in the township are not tarred. Many households in the township use well water in addition to communal water taps and there are high levels of poverty in the community. Chowa Township is equally close to the mine as Kasanda but is least affected by dust as it lies on the windward side of the mine. In contrast to Makululu, houses in Kasanda and Chowa are made of concrete bricks and use indoor tap water. Children from these townships were selected because soil samples in these townships are highly polluted with Pb (9-51188 mg/kg) and other metals (Nakayama et al. 2011).

2.2 Blood collection

The study was approved by the University of Zambia Research Ethics Committee (UNZAREC) and the Ministry of Health, Zambia. After informed and written consent was obtained from the children’s parents or guardians, blood samples were collected by qualified laboratory technicians at Chowa, Kasanda and Makululu clinics. Before sampling commenced, an awareness campaign about the research activities was conducted by community health workers in each township to encourage parents/guardians to take their children under the age of 7 to the health centers for sample collection. To avoid sample contamination, all blood collection supplies were kept in plastic ziploc storage bags before sample collection. For each child, data on the age, sex and residential area were recorded. Blood up to 10 mL was collected from the cubital vein of each child, after careful cleaning and sanitization of the venipuncture site with an ethanol swab to avoid contamination, into
plain blood collection tubes for Pb analysis. The blood samples were immediately stored in freezers at -20 °C after sampling and then transported in cooler boxes on dry ice to the laboratories of the Kabwe District Health Offices and Kabwe Provincial Veterinary Offices where they were again stored at -20 °C. After obtaining the material transfer agreement (MTA) clearance from the Zambia National Health Research Ethics Committee (NHREC), the blood samples were transported to Japan in cooler boxes on dry ice and analyzed for metal concentrations in Laboratory of Toxicology, Graduate School of Veterinary Medicine, Hokkaido University.

2.3 Sample preparation and metal extraction

All laboratory materials and instruments used in metal extraction were washed in 2 % nitric acid (HNO₃) and oven dried. The metal was extracted in blood samples using microwave digestion system (Speedwave MWS-2; Berghof) according to the manufacture’s instruction. Metal extraction was done as recommended by Schweitzer and Cornett (2008). Briefly, 1 mL of each blood sample was placed in prewashed digestion flasks, and 5 mL of 60 % nitric acid (Kanto Chemical) and 1 mL of 30 % hydrogen peroxide (Kanto Chemical) were added. After digestion in the microwave for 52 minutes and temperatures of up to 190 °C, the digested samples were transferred into plastic tubes. The volume was then made up to 10 mL with bi-distilled and de-ionized water (Milli-Q).

2.4 Metal analysis

Blood Pb concentrations were analyzed by Inductively Coupled Plasma-Mass Spectrometer (ICP-MS; 7700 series, Agilent technologies, Tokyo, Japan). The precision and accuracy of the applied analytical method was evaluated by analyzing the recovery rate using digested blood samples and spiking Pb standard solutions. Using this method, a good recovery of 97% was obtained. Certified Reference Materials, DORM-3 (Fish protein,
National Research Council of Canada, Ottawa, Canada) and DOLT-4 (Dogfish liver, National Research Council of Canada, Ottawa, Canada) were used to evaluate recoveries. Replicate analysis of these reference materials also showed good recoveries (95-105%). Instrument detection limit was 0.001 µg/L.

2.5 Statistical analysis

The data of BLLs were log transformed to stabilize variances. Statistical analysis was performed using JMP version 9 (SAS Institute, USA). The data are presented as mean, median and minimum-maximum values in µg/dL, wet weight. A stacked histogram was used to analyze blood Pb accumulation trends in Kasanda and Makululu as well as in boys and girls. Stepwise multiple linear regression analyses on log-transformed data were used to estimate the influence of area, sex and age (0 – 3 years and 4 – 7 years old) on BLLs. Correlations between age and BLL were analyzed by both linear and quadratic regression analysis. Samples from Chowa were not included in the comparisons due to smaller sample size compared to Kasanda and Makululu. A p-value of less than 0.05 was considered to indicate statistical significance.

3. Results

3.1 Blood lead levels (BLLs)

A total of 246 blood samples were collected from children, up to 7 years old, at Chowa (n = 17 samples), Kasanda (n = 100) and Makululu (n = 129) health centres. Concentrations of Pb in blood samples are shown in Table 1. As shown in Table 2, all of the sampled children had BLLs exceeding the guideline value that raise ‘health concerns’ (5 µg/dL). Numbers of children exceeding guideline values for
initiating chelation therapy (45 µg/dL), toxicity level (65 - 149 µg/dL) and levels associated with encephalopathy and death (> 150 µg/dL) are also shown.

3.2 Blood Pb accumulation patterns

Using a stacked histogram, blood Pb accumulation patterns in children from Kasanda and Makululu as well as concentration differences between boys and girls in the two townships were analysed (Figure 2). Blood accumulation differences were highlighted as the highest BLLs were seen in younger children (0 – 3 years) than children aged 4 – 7 years (Figure 3).

3.3 Age and Sex differences

Stepwise multiple linear regression analyses were performed on log-transformed data to estimate the influence of independent variables (age as continuous variable, sex represented as 0 for girls and 1 for boys, location (area) represented as 0 for Makululu and 1 for Kasanda) on BLLs (Table 3). Concentrations in children from Kasanda were higher than levels in children from Makululu (p < 0.05). There was no difference in the BLLs between boys and girls from Kasanda whereas in children from Makululu, BLLs were higher (p < 0.05) in boys than girls. Younger children aged 0 - 3 years accumulated higher concentrations of Pb in blood than children aged 4 – 7 years in both Kasanda and Makululu (p < 0.05).

Combining the data of Kasanda and Makululu, significant negative correlations between age and BLL were observed by both linear and quadratic regression analysis. Peak BLLs were observed around the age of 2 years (data not shown).
5. Discussion

The current study has demonstrated alarming childhood Pb poisoning in Zambia’s Kabwe town, revealing serious Pb exposure in the children under the age of 7 years in townships surrounding the closed Pb-Zn mine. The study analysed BLLs in children because it is well established that children are more vulnerable to Pb poisoning and sensitive to its neurotoxic effects than adults (Lidsky and Schneider 2003). All of the sampled children in the current study had indications of Pb poisoning, with BLLs exceeding the 5 μg/dL “level of concern” set by CDC (2012). Moreover, the current study revealed that children in these townships could be at serious risk of Pb toxicity as 18% of the sampled children in Chowa, 57% (Kasanda) and 25% (Makululu) had BLLs exceeding 65 μg/dL; the threshold widely considered to result in Pb toxicity (CDC 2002; Needleman 2004). Of the 246 children in the current study, 8 had BLLs exceeding 150 μg/dL, up to 427 μg/dL.

These findings agreed with reports in an earlier study before closure of the mine, where mean BLLs of 37 - 107 μg/dL were recorded in children from Kasanda Township (Clark APL, unpublished data). Of the 91 children between the ages of 1 - 2 years that were attended to at Kasanda clinic in the earlier study, 89% were reported to have accumulated BLLs > 60 μg/dL (Clark APL, unpublished data) compared to 61% of the sampled children from the same clinic in the current study. Therefore, there could be no difference between the severity of Pb poisoning during active mining period and almost 20 years after closure of the mine. Higher BLLs than the current study were recorded in children under the age of 5 years in Zamfara State in Nigeria, where the affected families processed metals in their dwellings (Blacksmith Institute 2010; Dooyema et al. 2012; Lo et al. 2012). In the study by Dooyema et al. (2012), BLLs exceeding 10 μg/dL were reported in all the 204 sampled
children in Nigeria. In children from Nigeria, mean BLLs (107.5 – 153.3 µg/dL) were higher than mean BLLs in the current study (39 – 82.2 µg/dL). However, the maximum BLL of 445 µg/dL recorded in children from Nigeria was comparable to that of the current study (427.8 µg/dL). Although data on mortalities due to Pb poisoning in Kabwe are scarce, clinical signs consistent with Pb poisoning such as anemia, small stature and weakness were observed in children from the sampled areas during the current study. In Nigeria, over 400 children were reported to have died of Pb poisoning (Blacksmith Institute 2011, Dooyema et al. 2012; Lo et al. 2012). Findings in the current study were higher than BLLs in children from an urban population in Kinshasa, Democratic Republic of Congo, where mean BLLs of 9.9 µg/dL and maximum concentrations of 49.3 µg/dL were recorded (Tuakuila et al. 2013). Moreover, BLLs in the current study were higher than mean BLLs (16.38 µg/dL) in children in the vicinity of Pb mines and sheltering plants in China (Lin et al. 2011). When compared to most European countries where the median BLL in the general population is below 5 µg/dL (Taylor et al. 2007), it is evident from the current study that levels of Pb poisoning in Kabwe, Zambia are alarming.

When the severity of Pb poisoning among the townships was compared in the current study, the mean BLL in children from Kasanda (82.2 µg/dL) was higher (p < 0.05) than Makululu (57.1 µg/dL). Kasanda and Makululu were subjected to atmospheric Pb pollution emanating from the neighbouring mine as they are located on the western side of the mine, which is in the direction of the prevailing winds. However, the difference in BLLs in children from the two townships could be attributed to distance from the mines. Although all these townships were close to the mine, some households in Kasanda (even Chowa) were within 1 kilometre of the mine and the abandoned mine dumps hence most of the polluted dust settles in Kasanda Township. Despite being further away from the mine
compared to Kasanda, Makululu Township, the largest shanty compound in Zambia equally poses a serious threat as roads, dwellings and house floors are dusty. Therefore, more children in Makululu Township could be at risk of Pb poisoning due to poverty and poor living conditions.

There was no gender difference in BLLs between boys and girls in Kasanda Township. This finding was in agreement with observations in the Democratic Republic of Congo (Tuakuila et al. 2013). However, trends in blood Pb accumulations between boys and girls were observed in the current study as boys in Makululu Township accumulated higher BLLs ($p < 0.05$) than girls in the same township. The same was observed when data of both Kasanda and Makululu were combined. Different behaviours between boys and girls could be one of the factors contributing to this difference as boys are likely to cover more distance away from home and play near the mine dumps than girls. When children in the current study were grouped according to age, it was observed that younger children between the ages of 0 – 3 years accumulated higher BLLs than their older counterparts (4 – 7 years). Significant negative correlation between age and BLL supported this finding. Similarly, younger children (1 – 2 years) in the Democratic Republic of Congo accumulated higher BLLs than older children (Tuakuila et al. 2013). Therefore, findings in the current study emphasized the increased susceptibility of younger children to the health risks of Pb pollution.

Earlier studies also observed that BLLs tend to peak at around 2 years of age (Koller et al. 2004). This observation is not unexpected as this period encompasses both the onset of independent ambulation and the time when a child’s oral exploration of the environment including hand-to-mouth or object-to-mouth behaviour (pica) is greatest. This exposure pathway of children has been well documented in other studies (Lanphear and Roghmann
It has been established that children typically ingest an average of 50 mg/day of soil (Stanek and Calabrese 1995). However, this amount can exceed 5 g a day in the case of pica (Mielke and Reagan 1998), with some children having been reported to ingest 25-60 g during a single day (Calabrese et al. 1997). Given that maximum Pb concentration in soils in the vicinity of the mine in Kabwe is about 50,000 µg/g or 50 mg/g (Nakayama et al. 2011), it means that children who ingest about 5 - 60 g of soil/day in the vicinity of the mine in Kabwe would ingest 250 - 3000 mg of Pb/day. Since the permissible tolerable weekly intake (PTWI) of Pb is 25 µg/Kg of body weight per week (WHO 1987), concentrations of Pb ingested by children through pica in Kabwe mining area could be high.

The current study has demonstrated that childhood Pb poisoning in Zambia’s Kabwe mining town is among the highest in the world, especially in children under the age of 3 years. Lead exposure among children is associated with developmental abnormalities including impaired cognitive function, reduced intelligence, impaired hearing and reduced stature (Canfield et al. 2003; Jusko et al. 2008). Although reports of clinical cases and deaths due to Pb poisoning among children in Kabwe are rare, the findings of the current study indicate that more studies need to be done in order to clearly establish the health effects of Pb poisoning in children exposed to Pb pollution in the townships around the mine in Kabwe. This is important because BLLs in all of the sampled children in the current study exceeded 5 µg/dL. In children, it has been established that neurobehavioral effects such as decrease in IQ may occur at BLLs < 10 µg/dL (Canfield et al. 2003). Moreover, BLLs of 40 - 60 µg/dL are considered to be markedly elevated, resulting in distinct neurobehavioral effects (TNO 2005). Since 18 % of the sampled children from Chowa, 57 % (Kasanda) and 25 % (Makululu) in the current study had markedly elevated
BLLs exceeding 65 μg/dL, it would not be surprising to observe neurological effects of Pb poisoning in the exposed children. Although this is the first published study evaluating Pb poisoning in Kabwe, it was earlier reported that during the mining period between 1971 to 1973, cases of suspected Pb poisoning with encephalopathy occurred among children aged 10 to 30 months living in the township of Kasanda (Clark APL, unpublished data). Therefore, the children in Chowa, Kasanda and Makululu townships should be closely monitored to enable early detection of clinical signs related to Pb toxicity and medical intervention.

6. Conclusions

Given that Pb poisoning among children in Kabwe was extensive, it is recommended that chelation therapy be commenced in the children with BLL exceeding 45 μg/dL prior to the onset of symptoms to reduce morbidity and prevent mortality in the affected children. This can be achieved for each child by devising and implementing an individualized plan of follow-up, especially for those children with extremely high BLLs. Interrupting the process of Pb poisoning through early detection and intervention can prevent children from dying or suffering severe permanent effects of Pb toxicity such as persistent seizures and mental retardation. Moreover, urgent interventions are required to reduce Pb exposure in the affected townships. This can be done through community-based programs to educate the affected communities about the health effects of Pb, sources of Pb and practical ways of reducing Pb exposure in their homes and communities.
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Conflict of interest

The authors declare no conflicts of interest.
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Figures legends:

Fig. 1. A map of Kabwe showing different geographic areas and sampling sites.

Fig. 2. Stacked histogram showing blood lead accumulation trends in children from Kasanda (46 boys and 54 girls) and Makululu (59 boys and 70 girls) townships of Kabwe, Zambia.

Fig. 3. Histogram showing blood lead accumulation trends in younger (0 – 3 years) and older (4 – 7 years) children from Kasanda and Makululu townships of Kabwe (Zambia).
Table 1.
Mean age (year) and BLLs (µg/dL) of children from Chowa, Kasanda and Makululu townships in vicinity of the Pb-Zn mine in Kabwe, Zambia

<table>
<thead>
<tr>
<th>Township</th>
<th>Mean age</th>
<th>Sample size</th>
<th>Arithmetic mean BLL</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chowa</td>
<td>5.76</td>
<td>n = 17</td>
<td>39.0</td>
<td>39.3</td>
<td>15.6</td>
<td>79.7</td>
</tr>
<tr>
<td>Kasanda</td>
<td>3.65</td>
<td>n = 100</td>
<td>82.2</td>
<td>74.9</td>
<td>5.40</td>
<td>427.8</td>
</tr>
<tr>
<td>Makululu</td>
<td>4.51</td>
<td>n = 129</td>
<td>57.1</td>
<td>51.1</td>
<td>9.40</td>
<td>388.7</td>
</tr>
</tbody>
</table>

\( n = \text{Number of samples} \)
Table 2.

Numbers of children (under the age of 7 years) with elevated BLLs from the sampled townships in Kabwe

<table>
<thead>
<tr>
<th>Reference limits</th>
<th>Chowa $(n = 17)$</th>
<th>Kasanda $(n = 100)$</th>
<th>Makululu $(n = 129)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 5 µg/dl</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5 - 44 µg/dL – elevated levels</td>
<td>8</td>
<td>27</td>
<td>50</td>
</tr>
<tr>
<td>45 - 64 µg/dL – initiate treatment</td>
<td>7</td>
<td>15</td>
<td>44</td>
</tr>
<tr>
<td>65 - 149 µg/dL – toxicity level</td>
<td>2</td>
<td>50</td>
<td>33</td>
</tr>
<tr>
<td>&gt; 150 µg/dL – encephalopathy, death</td>
<td>0</td>
<td>8</td>
<td>2</td>
</tr>
</tbody>
</table>

$n =$ Number of children sampled
Table 3.

Blood lead accumulation differences (age, sex and site) by stepwise multiple linear regression analyses in children from Kasanda and Makululu townships in Kabwe.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>nDF</th>
<th>SS</th>
<th>F Ratio</th>
<th>p value (Prob&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kasanda and Makululu</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>1.898</td>
<td>1</td>
<td>0.00</td>
<td>0.00</td>
<td>1.0</td>
</tr>
<tr>
<td>Age</td>
<td>-0.035</td>
<td>1</td>
<td>1.05</td>
<td>15.84</td>
<td>9.35E-05</td>
</tr>
<tr>
<td>Sex(F-M)</td>
<td>-0.050</td>
<td>1</td>
<td>0.56</td>
<td>8.44</td>
<td>0.004</td>
</tr>
<tr>
<td>Area(Makululu-Kasanda)</td>
<td>-0.048</td>
<td>1</td>
<td>0.48</td>
<td>7.25</td>
<td>0.008</td>
</tr>
</tbody>
</table>

| Kasanda                       |          |     |     |         |                 |
| Intercept                     | 1.936    | 1   | 0.00| 0.00    | 1.0             |
| Age                           | -0.033   | 1   | 0.41| 4.36    | 0.039           |
| Sex(F-M)                      | -0.060   | 1   | 0.35| 3.77    | 0.055           |

| Makululu                      |          |     |     |         |                 |
| Intercept                     | 1.861    | 1   | 0.00| 0.00    | 1.0             |
| Age                           | -0.038   | 1   | 0.66| 14.3    | 0.0002          |
| Sex(F-M)                      | -0.043   | 1   | 0.22| 4.79    | 0.030           |

Bold indicate significant (p < 0.05), nDF: number of degrees of freedom for a term, SS: Sequential Sum of Squares
Fig. 2
Fig. 3