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# Determinants of Primary Pyomyositis in Northern Uganda

# David Lagoro Kitara<sup>1\*</sup>, Paul Okot Bwangamoi<sup>2</sup>, Henry Wabinga<sup>3</sup> and Michael Odida<sup>3</sup>

<sup>1</sup>Department of Surgery, Faculty of Medicine, Gulu University, Gulu, Uganda. <sup>2</sup>Department of Pharmacy, Faculty of Medicine, Gulu University, Gulu, Uganda. <sup>3</sup>Department of Pathology, College of Health Sciences, Makerere University, Uganda.

# Authors' contributions

This work was carried out in collaboration between all authors. Author DLK designed the study, wrote the protocol, and wrote the first draft of the manuscript. Authors POB, HW and MO managed the literature searches, analyses of the data and performed the Histological. All authors read and approved the final manuscript.

#### Article Information

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# ABSTRACT

Aim: To describe the determinants of primary pyomyositis in Northern Uganda. Study Design and Setting: A case-control and a cohort study designs were conducted in

Hospitals in Northern Uganda. **Methods:** Primary pyomyositis patients were consecutively recruited and followed to discharge. Controls had minor trauma and were age and sex matched with cases. Patients were admitted, investigated (clinical features, imaging, hematology, clinical chemistry and histology from muscle biopsy); managed surgically and followed up to discharge. Those that did not meet the inclusion criteria for diagnosis histologically were excluded. Ethical approval was obtained from Gulu University IRB. **Results:** The determinants of primary pyomyositis were: HIV positivity with low CD<sub>4</sub> counts (<250 cells/ml) ( $\chi^2$ =11.748; p<0.001; aOR 11.292 at 95% CI (0.698,182.707) (p=0.088)); clinical features of immunosuppression/AIDS ( $\chi^2$ =12.70; p<0.001; aOR 6.50 at 95% CI (0.000,2.500) (p=0.850)); High serum creatinine level ( $\chi^2$ =20.191; p<0.001; aOR 6.070 at 95% CI (0.289,127.545) (p=0.317)) and Low serum albumin (malnutrition) ( $\chi^2$ =103.247; p<0.001; aOR 226.004 at 95% CI (13.449, 3797.786) (p<0.001)).

**Conclusion:** The determinant of primary pyomyositis was low serum albumin (malnutrition) while clinical features of immunosuppression/AIDS, high serum creatinine level and HIV positivity with low CD<sub>4</sub> counts were risk factors but not independent predictors of this disease.

Keywords: Determinants of primary pyomyositis; clinical presentations; Gulu; Uganda.

#### 1. INTRODUCTION

Pvomvositis is a suppurative infection of skeletal muscles which is clinically characterized by localized muscle pains, swelling and tenderness [1-5]. It was described initially as an infection mainly found in the tropics, although it has now been recognized in temperate climates with increasing frequency of HIV/AIDS infected persons [6-10]. The disease was first described by Scriba, a German in 1885 while on a consultancy to the Japanese Government and it has been primarily recognized as a disease occurring in the young and relatively healthy persons [11] although cases were later on reported in persons of all ages [12]. Most pyomyositis patients in tropical regions were otherwise healthy individuals without underlying co-morbidities, while most patients in temperate regions were immuno-compromised or had other serious underlying medical or surgical conditions [6,12].

Several unproven hypotheses have attempted to connect the demographic factors associated with pyomyositis to such tropically predisposed circumstances such as malnutrition [13,14], protozoan infection [15,16] and disordered immunity [17,18]. Studies have observed no epidemiological difference between pyomyositis in the temperate climate and those in the tropics [19].

It was therefore observed that pyomyositis could involve any muscle group in the body; a single muscle was usually affected although 11-45% of patients had involvement of multiple sites [20-24]. In a review of 676 cases of pyomyositis, Bickels et al. [25] identified 112(16.6%) patients with multiple site involvement. Since then, several studies have confirmed the same finding and noted specifically that pyomyositis involved the largest muscle groups located around the pelvic girdle and lower limbs [26-28].

llio-psoas pyomyositis was long considered to be the commonest form of this infection however, most of these cases were found to be secondary infections that had developed as an extension from the adjacent tuberculosis in the spine or iliac lympnodes [29,30]. Today, secondary iliopsoas pyomyositis is most commonly associated with gastrointestinal or urinary tract infections [15,31-33]. The bacteria causing this infection were frequently not isolated from blood cultures or cultures of purulent materials [25]. In a study of 452 primary pyomyositis patients in whom the offending bacteria was identified, 350(77%) had a positive culture of *Staphylococcus aureus* [23,34-40].

There is wide array of differential diagnosis for pyomyositis including fever of unknown origin (FUO), muscle contusion, septic arthritis, bursitis, rheumatoid disease, osteomyelitis, cellulitis, muscle hematoma, deep venous thrombosis (DVT), muscle rupture or muscle strain, osteosarcoma of muscle, rhadomyosarcoma, polymyositis leptospirosis. trichinosis. and pyomyositis of the right ilio-psoas muscle which may be confused for acute appendicitis [10,41]. These wide arrays of differential diagnoses for pyomyositis showed how pyomyositis could easily be confused with other different conditions, thus it could also present atypically, a factor which has led to misdiagnosis and delayed diagnosis of many cases.

The objective of this study was to describe the determinants of primary pyomyositis in patients who presented with muscle pains and swelling in Northern Uganda in order to provide clear method of differentiating the atypical presentations that provided diagnostic dilemma to clinicians in this region.

#### 2. MATERIALS AND METHODS

#### 2.1 Study Designs

This was a case-control and a prospective cohort study designs carried out on primary pyomyositis patients and their controls.

# 2.2 Study Sites

This study was conducted in Hospitals in Gulu, Kitgum, Agago and Lamwo districts in Northern Uganda from January 2011 to September 2013. These districts form the central northern Uganda which is just recovering from over 20 years of civil war between the Government of Uganda and the rebel, Lord's Resistance Army (LRA). Gulu Regional Hospital is situated in one of the regional centers for northern Uganda and draws largely rural population; many of whom lived in internally displaced people camps (IDPS) for the period between 10 to 12 years for safety from the insurgency. During the encampment, the population was fed exclusively of food obtained from United Nations World Food program (UNWFP) which was reported to have provided only 60% of the required calories per day. According to the Uganda Bureau of statistics, the 4 districts where the study was conducted have a total population of about 1,200,000 people [42].

# 2.3 The Study Population

The study population was primary pyomyositis patients who were recruited consecutively from those who sought treatment from these hospitals. Controls were age and sex matched with cases and had reported to these same hospitals with minor trauma. The selection of the study population was conducted by the Principal Investigator and his team after ascertaining that they fulfilled the inclusion criteria. Symptoms and signs of primary pyomyositis, the investigations including imaging modalities, haematology, biochemistry and clinical chemistry were used to obtain the test variables and to confirm diagnosis. Primary pyomyositis was confirmed by a histological diagnosis of muscle biopsy. Surgical management by incision, drainage and debridement (I,D&D) was conducted by the research team. The controls underwent clinical evaluation and blood draw for haematology, biochemistry and clinical chemistry.

#### 2.4 Inclusion Criteria for the Patients

Primary pyomyositis patients who were 13 years and above and had obtained informed consent from their parents and assent for children 14 years and above and had lived in the region for 2 years.

# 2.5 Exclusion Criteria for the Patients

Pyomyositis with other suppurative infections in the neighbouring structures.

# 2.6 Inclusion Criteria for the Controls

Age and sex matched patients that reported to the same health facility with minor trauma within 24 hours of the occurrence and had obtained informed consent from their parents and assent for children 14 years and above and had lived in the region for 2 years.

# 2.7 Exclusion Criteria for the Controls

Those with minor trauma and had reported to the same health facility 24 hours after the occurrence of the trauma.

#### 2.8 Study Procedures

For each patient, a control was recruited; they underwent a thorough clinical evaluation including history and physical examinations; the vital signs such as pulse rate, temperature, blood pressure and heart rate were measured. Each patient and control had a blood draw for haematological investigation (using Cyflocounter clinical machine-Sysemex-XS-1000i (USA), chemistry (Huma Star 180, Germany) and analysis biochemical (HumaLyte Plus<sup>3</sup>. Germany) and the  $CD_4/CD_8$  cell counts using (Model: BD Facs caliber<sup>TM</sup>, Flow cytometer, 342975, Germany) and for HIV status using (Screening-Determine, confirmatory-stat-Park and tie-breaker-unigold) and confirmed by Polymerase Chain Reaction (PCR). Primary pyomyositis patients underwent surgical procedures (Incision. Drainage and Debridement) and muscle biopsy was taken for histological analysis. Pus from muscle abscesses was cultured and was tested for antibiotics susceptibility using Kirby-Buer disk diffusion method on 4% Salted Mueller Hinton II agar for Methicillin and non salted Mueller Hinton II agar for the common antibiotics (NCCLS M100S9).

#### **2.9 Anthropometric Measurements**

For each case and control, weight was measured using a standardized weighing scale and

measured to (0.1 Kg). Height was measured when the participant was standing in an erect position, bare footed on a stadiometer with a movable head piece. The head piece was leveled with skull vault & height was recorded to the nearest 0.5 cm. The BMI was then calculated using the formula BMI=weight (Kg)/Height<sup>2</sup> (Meter<sup>2</sup>). Each person's BMI was then graded according to the WHO (2007) classification to categorize it into: BMI <18.5 as Under Weight; BMI 18.5-24.5 as Healthy weight range; BMI 25-30 as Overweight (grade 1 obesity); BMI >30-40 as Obese (grade 2 obesity); BMI >40 as Very obese (morbid or grade 3 obesity).

# 2.10 Data Analysis

Statistical software package (SPSS) version 15.0 (Chicago, IL, USA) was used for univariate analysis of socio-demographic characteristics and other variables. Bivariate analysis was used to test the associations between the outcome and independent variables. Odds Ratios (OR) with a 95% confidence interval (CI) was calculated to determine the risk factors in primary pyomyositis patients. Categorical variables were analyzed using a Chi-square tests and Fisher's exact t-test were used where cell numbers were expected to be less than five. A multivariable Logistic Regression analysis was conducted to determine the determinants of primary pyomyositis. A p-value of less than 0.05 as the cut off for the level of statistical significance was used

# 2.11 Ethical Consideration

The study was approved by the Research and Ethics Committee of Gulu University Medical School and Uganda National Council of Science and Technology (UNCS&T) and was conducted in accordance with the principles of Good Clinical practice and standards. All parents/guardians of the participants gave a written informed consent and confidentiality of information was maintained throughout the study and follow-up of the patients.

# 3. RESULTS

Sixty three primary pyomyositis patients were recruited for the study; 11 patients were HIV positive while 52 patients were HIV negative. The modal age group for the patients was 10-19 years which had a total of 29 persons. The age range of the patients in the study population was wide and was ranging between 13 to 65 years.

#### 3.1 The Age Distribution

The majority of primary pyomyositis patients were in the young age groups: 29(46.03%) in 10-19years; 18(28.57%) in 20-29 years; 11(17.46%) in 30-39 years; 3(4.76) in 40-49 years; 1(1.59%) in 50-59years; 1(1.59%) in >60 years. The mean age for the patients was 22.98 years (SD+10.667).

#### 3.2 HIV Positive Status

Eleven out of sixty three (17.5%) primary pyomyositis patients were HIV positive; while there were ten HIV positive among the control group with a sero-prevalence of 10/63 (15.9%). All HIV positive primary pyomyositis patients and controls did not know their HIV status and were ARV naïve. The matched analysis produced an unadjusted Odds ratio of 1.121 and the maximum likelihood estimate OR (MLE) of 1.121 (Exact 95% confidence limits for MLE, 0.439<OR<2.864) at bivariate analysis (Table 3). However, when this variable was subjected to a multivariable logistic regression analysis, an adjusted OR of 11.292 at 95% CI (0.698, 182.707) (p=0.088) was obtained (Table 3). Meaning there was an association between HIV positivity with primary pyomyositis but this did not reach statistical significance at 0.05 and therefore HIV positive status was not an independent predictor of primary pyomyositis.

#### 3.3 Low Serum Albumin Level

The prevalence of low serum albumin among the cases was 59/63(93.7%) and their mean serum concentration was 34.48 mg/dL albumin (SD+3.34) which was far lower than normal limit of serum albumin level compared to their matched controls with only 2/63(3.2%) with low serum albumin which had the overall mean of 52.59 mg/dL (SD+2.45) (Table 1). The Chisquare test ( $\chi^2$ =103.247; p<0.001) showed a statistically significant association between low serum albumin and primary pyomyositis and the adjusted OR of 226.004 at 95% CI (13.449, 3797.786) (p<0.001) was obtained (Table 3). Thus, there was an association between primary pyomyositis and low serum albumin and this was statistically significant at 0.05. Therefore, low serum albumin increased the risk of developing primary pyomyositis in the study population by 230 times and low serum albumin was an independent predictor of primary pyomyositis (Table 3).

#### 3.4 Low Haemoglobin Concentration

Most primary pyomyositis patients 29/63 (46.03%) had low haemoglobin concentration (<10g/dl) while their controls had 4/63(6.35%). There was an association between low haemoglobin concentration and primary pyomyositis patients ( $\chi^2$ =25.66; p<0.001) and unadjusted OR 12.581 at 95% CI (4.075, 38.843) and a Fisher's exact t-test (p<0.001) at bivariate

analysis. However, when this variable was subjected to a multivariable logistic regression analysis, an adjusted OR of 0.622 at 95% CI (0.130, 30.858) (p=0.845) was obtained. Meaning there was an association between low haemoglobin concentrations with primary pyomyositis at bivariate analysis but was not an independent predictor of primary pyomyositis (Tables 2 and 3).

#### Table 1. Shows the study variables of primary pyomyositis at univariate analysis

Variables	Mean	SD	Median	Minimum	Maximum
LoS (days)	30.00	2.34	30.00	2.00	365.00
DoHS (days)	10.95	3.76	11.00	2.00	22.00
Temp (°C)	37.86	0.70	37.90	36.00	39.20
Pulse rate	99.38	17.34	100.00	56.00	158.00
Systolic BP	110.00	15.84	110.00	82.00	170.00
Diastolic BP	71.60	15.84	70.00	30.00	120.00
Resp. Rate	24.25	5.80	25.00	15.00	45.00
Haemoglobin (Hb)	9.72	2.40	10.20	1.20	16.30
WBC count	8.25	3.66	7.40	3.54	20.91
Serum creatinine	0.67	0.07	0.70	0.50	0.90
Serum Na⁺	141.03	3.15	141.00	132.50	147.50
Serum K <sup>⁺</sup>	4.28	0.34	4.30	3.50	5.40
Serum albumin	34.48	3.34	34.00	28.00	52.00
CD <sub>4</sub> /CD <sub>8</sub>	224.00	92.68	213.00	123.00	411.00
Viral Load	3,605.73	3,131.20	2,346	897.00	10,345

BP=Blood Pressure; Systolic pressure (100-120 mmHg); Diastolic pressure (60-80 mmHg); CD=Cluster of Differentiation; SD=Standard Deviation; °C=Degree centigrade; Normal temperature (36.5-37.3°C); K+=Potassium concentration (3-5 mmol/l); Na+ = Sodium Concentration (135–145 mmol/l); WBC = White blood count (4-11x10<sup>3</sup> /ml); LoS = Length of symptoms (days); DoHS = Duration of Hospital stay (days)

# Table 2. Shows the case-control results between primary pyomyositis patients and controls at bivariate analysis

Variables	Cases (n=63)	Controls (n=63)	X <sup>2</sup>	P-value
Features of immunosuppression/AIDS	16	2	12.70	<0.001
High temperature (>37.3°C)	52	0	88.54	<0.001
Low haemoglobin (<10g/dl)	29	4	25.66	<0.001
High serum creatinine level (mmol/L)	37	28	20.19	0.001
HIV positive status	11	10	0.06	0.811
Low serum albumin level (<38g/L)	59	2	103.25	<0.001
Low CD₄/CD <sub>8</sub> counts (<250 cells/ml)	8	0	11.75	0.001

# Table 3. Shows the Multivariable Logistic Regression output for determinants of primary pyomyositis

Variables	Unadj (OR)	95% CI	Adj (OR)	95% CI
Features of	10.383	(2.275, 47.397)	6.500	(0.000, 2.500)
immunosuppression/AIDS				
High temperature	6.727	(3.900,11.605)	0.001	(0.000,∞)
Low haemoglobin	12.581	(4.075, 38.843)	0.622	(0.130, 30.858)
High serum creatinine level	8.429	(4.205, 16.890)	6.070	(0.289, 127.545)
Low serum albumin level	449.875	(79.382, 2549.540)	226.004	(13.449, 3797.786)
HIV positive	1.121	(0.439, 2.864)	11.292	(0.698, 182.707)

#### 3.5 High Serum Creatinine Level

The serum creatinine level was moderately high pyomyositis in most primary patients 37/63(58.7%) while their controls had 28/63(44.44%) with high serum creatinine level. The statistical analysis showed an association between high serum creatinine and primary pyomyositis and that this association was statistically significant ( $\chi^2$ =20.191; p=0.001) using chi-square test. The unadjusted OR was 8.429 at 95% CI (4.205, 16.89) at bivariate analysis. However, when this variable was subjected to a multivariable logistic regression analysis, an adjusted OR of 6.070 at 95% CI (0.289, 127.545) (p=0.317) was obtained. Meaning there was an association between low concentrations haemoglobin with primary pyomyositis at bivariate analysis but was not statistically significant at 0.05 at multivariable logistic regression analysis and therefore not an independent predictor of primary pyomyositis.

# 3.6 High Body Temperature (T>37.3°C)

Most primary pyomyositis patients 52/63(82.54%) presented with a high body temperature compared to none among controls and this difference was statistically significant  $(\chi^2 = 88.54; p < 0.001)$  using the chi square test and an unadjusted OR was 6.727 at 95% CI (3.700, 11.605) at bivariate analysis. However, when this variable was subjected to a multivariate logistic regression analysis, an adjusted OR of 0.001 at 95% CI (0.00,∞) (p=0.995) was obtained (Table 3). Meaning there was an association between low haemoglobin concentrations with primary pyomyositis at bivariate analysis but was not an independent predictor of primary pyomyositis at the logistic regression analysis.

#### 3.7 Features of Immunosuppression/AIDS

with clinical All patients features of immunosuppression/AIDS met the WHO, 2007 Clinical Case Definition (CCD) of AIDS and this was shown by (severe weight loss >10 kg, skin changes, signs and symptoms of severe bacterial and fungal infections). All sixteen primary pyomyositis patients had (low serum albumin level, low BMI and clinical features of malnutrition) while half of those were HIV positive. The association between clinical features of immunosuppression/AIDS and HIV positivity was statistically significant at 0.05 using the Chi square test and Odds Ratio (Table 2). All these HIV positive primary pyomyositis patients

with evident clinical features of immunosuppression/AIDS had low CD<sub>4</sub>/CD<sub>8</sub> counts and most of them had CD<sub>4</sub> counts which were less than 250 cells per ml. Furthermore, the adjusted Odds Ratio for patients with clinical features of immunosuppression/AIDS was 6.50 at 95% CI (0.000, 2.500) (p=0.850) (Table 3). Thus, there was an association between clinical features of immunosuppression/AIDS and primary pyomyositis but this did not reach statistic significance at 0.05 at multivariable logistic regression analysis. Meaning the clinical features of immunosuppression/AIDS was not an independent predictor of primary pyomyositis.

#### 3.8 Histological Findings of Muscle Biopsy

The muscles involved were necrotic with some parts containing muscle tissues that had undergone fibrous degeneration. There was mass infiltration of the muscle tissue with inflammatory cells of immune response, plenty of pus cells interspersed with giant cells. The blood vessels were collapsed and filled with thrombus and some had undergone fibrous degeneration.

# 3.9 Muscles Involved

This study found that the most commonly affected muscles with primary pyomyositis were Quadriceps femoris 24(34.8%), Gluteus maximus 14(20.3%), *Gastrocnemus* 13(18.84%); *Latissimus dorsi* 3(4.35%); *Biceps brachi* 3(4.35%) and others 8(12%) respectively.

#### 4. DISCUSSION

Pyomyositis has been considered a disease of the tropics that occurs primarily in young and relatively healthy persons [25,43]. Several unproven hypotheses have been proposed to explain this disorder including malnutrition, HIV/AIDS, protozoa infection, viral muscle infection, trauma and disordered immunity [7,8,25,44-47]. Muscles are normally remarkably resistant to suppurative infections and muscle trauma has been reported to be necessary before an experimentally induced bacteriaemia could cause pyomyositis in animals [48]. In humans, muscle abscesses are rarely a complication of severe *Staphylococcal* sepsis [49].

In this study, the information derived adds more information to previous studies conducted in the tropics which revealed that pyomyositis patients were generally young adults with few reported or confirmed cases of Diabetes mellitus, hypertension and bleeding disorders. However, HIV/AIDS infection with low  $CD_4$  count level is a common finding in primary pyomyositis patients and it is a risk factor which makes muscles of these patients susceptible to bacterial infection [6,10] (Tables 2 and 3). For those that were HIV negative, they all suffered from malnutrition to a level that some patients 8/52 met the WHO Clinical Case Definition (CCD) of AIDS.

The socio-demographic characteristics of the patients were comparable to most studies already conducted in the tropical region where large proximal lower limb and truncal muscles were the most commonly involved and the most commonly isolated organism was *Staphylococcus aureus*.

# 4.1 HIV Positive and Low CD<sub>4</sub>/CD<sub>8</sub> Counts

In a series of studies conducted in the tropical region, it has been observed that pyomyositis was a bacterial infection highly and significantly associated with HIV/AIDS infection and thus should be considered a strong sign of stage III-IV of the HIV disease [50,51]. This study further observed that the prevalence of HIV was 17.5% in cases compared to 15.9% in the control population and these figures reflected the similarities in the prevalence of HIV in the general population in northern Uganda [42]. Several factors have been postulated to be responsible for the higher prevalence of pyomyositis in HIV positive population. The mechanism by which HIV infection predisposed a patient to pyomyositis remains unclear but factors that have been suggested included: IgG2 deficiency [52]; altered phagocytosis [53]; primary HIV myopathy or antiretroviral therapy [54], immune compromise (low CD<sub>4</sub> counts) and increased rates of Staphylococcal carriage [6.55-57]. In all these studies, there was however no statistically significant differences in the sexes of pyomyositis patients in association with HIV infection [58,59], a fact that demonstrated that this disease was not a sex linked or sex preferred disease although the prevalence of HIV was higher in females compared to males in Uganda [60]. It was therefore noted that bacterial pyomyositis among HIV infected persons typically occurred in those patients with endstage acquired immunodeficiency syndrome [6]. Even in the HIV negative primary pyomyositis patients, more than half of the patients were malnourished. In conclusion therefore, HIV positivity with low CD<sub>4</sub> counts of less than 250

cells per ml was a risk factor for the development of primary pyomyositis although it was not an independent predictor of pyomyositis at multivariable logistic regression analysis.

# 4.2 Low Serum Albumin (Malnutrition)

There was a statistically significant association between low serum albumin and primary pyomyositis and the odds of developing primary pyomyositis were increased 230 times in patients with low serum albumin. Pyomyositis has been described in association with tropical climates and occasionally in association with parasites and nutritional deficiencies and it is suggested that they are possible aetiological factors [61,62]. Our study and those previously conducted in northern Uganda indicate a strong association between primary pyomyositis with nutritional deficiencies exhibited by a low serum albumin levels, clinical features of malnutrition and low Body Mass Index (BMI) [63]. It was further observed that, malnutrition in all its forms remained largely a hidden problem since majority children affected of were moderately malnourished or have micronutrient deficiencies that were not routinely assessed [64]. Malnutrition therefore remains a major public health problem in Uganda and particularly northern Uganda which has suffered for over 20 years of civil war in which the people lived in internally displaced peoples camps (IDPs) for over 10 years.

A study in Uganda observed that malnutrition compromised the immune system and increased the risk and severity of infections and diseases [65]. Kikafunda and others have shown that there was a co-existence of high prevalence of malnutrition in Uganda with infections among children, suggesting that poor immune function might be a result of inadequate nutrition [66]. Furthermore, four commonest infections including Malaria, Acute Respiratory Infections (ARIs), diarrhoeal diseases and HIV/AIDS in Uganda have been identified to be highly associated with malnutrition [66]. In this study therefore, malnutrition was shown to be an independent predictor of primary pyomyositis in the study population.

The authors however wish to acknowledge that there may have been some limitations to the study including small numbers of patients with HIV/AIDS in both cases and control. This may have led to the current conclusion however, the rigor of the study has demonstrated that malnutrition was the major factor in the development of primary pyomyositis in Northern Uganda.

#### 5. CONCLUSION

The determinant of primary pyomyositis was low serum albumin (malnutrition) while clinical features of immunosuppression/AIDS, high serum creatinine level and HIV positivity with low  $CD_4$  counts were risk factors but not independent predictors of this disease.

# CONSENT

All authors declare that written informed consent/Assent was obtained from each of the children in the presence of the guardian/parents.

# ETHICAL APPROVAL

All authors hereby declare that the research have been examined and approved by Gulu University, Faculty of Medicine Institutional Review Committee, which is the appropriate ethics committee and the approval have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. The Ethical clearance reference number is HS 922 and find attached the approval letter.

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#### **COMPETING INTERESTS**

Authors have declared that no competing interests exist.

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