Determinants of Keloid Recurrence in African Patients at Kenyatta National Hospital

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Declaration

I hereby declare that this thesis is my original work and has not been presented for examination in this or any other university.

Signed 

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This thesis has been submitted for examination with our approval as University supervisors

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Publications/ Manuscripts from the study

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3. Nangole F.W., Ouyang K, Anzala O, Ogeng'o J., Agak G.W. Multiple Cytokines Elevated in Patients with Keloids: Is It an Indication of Auto-Inflammatory Disease?. ***J Inflamm Res.* 2021; 14:2465-2470**

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7. Ferdinand W. Nangole, [1] Kelsey Ouyang, [2] Omu Anzala, [3] Julius Ongengo, [4] George W Agak: Familial and Sporadic keloids: A comparative study on Presentation, Management and Outcome. ***East African Medical Journal*; 2022;99;(5) ;69-74**

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Conference presentation

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   2nd International Keloid symposium; Rome 2018; June 7th -8th

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   Intra-lesional or Extra-lesional keloid excision?
   Wound care society of Kenya virtual conference 8th of Dec 2020

5. Nangole, F.W., Agak, G., Ogengo, J., Anzala, O.
   Does Keloid histology Influence recurrence
   Proceedings of the 4th international keloid symposium
   October 7-9th 2022, Montepellier , France

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   Determinants of keloids recurrence: the role of keloid recurrence scoring system
   Proceedings of the 4th international keloid symposium
   October 7-9th 2022, Montepellier, France

7 Nangole F. W., Agak G., Ogengo J., Anzala O.
   Is keloid an auto inflammatory disease?
   Proceedings of the 4th international keloid symposium
   October 7-9th 2022, Montepellier, France

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ACRONONYMS/ABBREVIATIONS

BC Before Christ

EGF Epidermal Growth Factors

EL Extra-lesional

HGF Hepatocyte growth factors

HLA Human Leucocyte Antigen

IF Inter-feron

IL Intra-lesional

IL 1RA Inter-leukin 1 receptor antigen

IL 2RA Inter-leukin 2 Receptor antigen

KR Keloid Recurrence

KAVICR Kenya AIDS Vaccine Initiative clinical research

KNH KenyattaNational Hospital

MCP Macrophage chemotactic protein

MIP Macrophage induction protein

MMPS Matrix Metallo-proteinases

MSC Mesenchymal stem cells

MSH Melanocyte Stimulating Hormone

NKR Non - keloid recurrence

PDGF Platelet Derived Growth Factors

TGFβ Transforming Growth Factors

TNF Tumor necrosis factor

UoN University of Nairobi

VEGF Vascular endothelial growth factor

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SUMMARY

Background: Keloids are fibro-proliferative disorders of the skin resulting in excessive tissue overgrowth usually due to minor tissue injury. Keloids are attributed to abnormal wound healing with an exaggerated inflammatory phase and a poorly controlled proliferative phase. Patients with keloids have been shown to be heterogeneous in clinical, pathological and genetic make ups. There are many modalities of treating keloids. However, all are associated with high recurrence rates. Factors that influence keloid recurrence either locally or globally have not been well demonstrated. This study therefore sought to determine factors that are associated with keloid severity and recurrence among patient's cohorts in KNH. A control of patients with no keloids nor family history of keloids were also followed up in the study.

Rationale for the study: Keloid disease is a common medical condition. It results in both psychological and physical stress to patients. The recurrence rates are high with studies quoting recurrence of between 10 and 70%. To the best of my knowledge, no studies had been done to determine factors that influence recurrence in our population. Understanding factors that influence keloid recurrence would aid in keloid management by enabling patients to have an informed mind on the chances of recurrence as well as allowing the physicians to start on second line management early in cases where recurrences are likely to be high.

Objective: To determine the genetic, clinical, inflammatory and histo-pathological factors that influence keloid recurrence.

Study Design: A Case control observational study of patients presenting with keloids at the KNH.

Setting: Kenyatta National Hospital, Nairobi, Kenya.

Ethical considerations: This study was approved by the UON/KNH ethics and research committee. Confidentiality ran supreme during the course of the study. Informed consent was sought from all patients who participated in the study**.**

Methodology: Patients with keloids and a control with no keloids after consenting were recruited for the study. Inclusion in the study was limited to patients whose keloids could be excised and closed primarily. Excluded from the study were patients with extensive keloids that could not be closed primarily after excision or those with chronic medical conditions. Targeted

history and physical examination on the causation of keloids and clinical presentation was taken. Patients were managed by surgical excision followed by superficial radiotherapy. Specimens from the excised keloids were analyzed for lymphocytes, macrophages, mast cells, fibroblasts as well as extracellular matrix density. Immuno-histochemical studies were done on selected slides to determine the presence of mast cells, fibroblasts and myofibroblasts. Mesenchymal stem cells were assessed by flow cytometry studies. The extra-cellular matrix was analyzed for vascularity and the collagen bundles densities. Blood samples were taken for blood groups, HLA sub-types and inflammatory cytokines. Post-operatively, patients were followed up to a minimum of one year and any keloid recurrences were noted.

Data and Statistical Analysis: All the information was collected in a pre-tested questionnaire. A student T-test was used to compare means and a Chi-square was used to test variance from the mean. Pearson's correlation co-efficient (r) was used to measure the strength of the association between the various variables and keloid recurrence. Analysis of variance (ANOVA) was done to determine variance in the various groups. Bonferroni correction was done to determine the significance of the statistical differences. Multiple regression analysis was done to determine the strength of variables in keloid recurrence. Descriptive analysis, cross tabulation and comparison of means were used in analysis. Odds ratios and mean difference were used to measure association and 95% confidence intervals to measure significance. STATA/SE statistics/data analysis version 10 software was used for analysis.

Results: A total of 90 patients and a control of 59 patients were recruited for the study. The male to female ratio for the keloid and control patients was 1:2. The age ranges for the keloid patients was 15 to 65 years with a mean age of 29.5 and a median age group of 20-25 years. Familial keloids contributed to 53% of keloids with sporadic keloids at 47 %. Majority of the keloids were due to trauma (63.5%). Ear keloids were the most common accounting for 43% of the keloids. Male patients had a significantly higher recurrence rate of 31% compared to the female patients at 12%. The recurrence rates were higher in the familial keloids at 26.9 % compared to the sporadic keloids at 18.5%. There was no statistically significant difference in the surface area of patients who had keloid recurrence and those with no recurrence. Keloid patients with blood group A were more prone to recurrence compared to the other blood groups. Patients with keloids had a significantly higher positive alleles of DQA*01, DQB1*05, DQB1*06 and DRB1*15. Comparison between patients who had keloid recurrence and those who did not

revealed DQB1*06 to be significantly elevated in patients with keloids recurrence (p-value <0.05). Keloid specimens had significantly higher MSC counts compared to the normal skin. Further Keloid specimens had a higher ratio of MSC compared to the normal skin. Histological parameters revealed that high macrophages, lymphocytes and fibroblast counts were significantly associated with keloid recurrence.

Conclusion: This study demonstrates that keloid recurrence is influenced by many factors including patients′ sex, history, clinical presentation, keloid histology and HLA subtypes. There is need to identify these factors in patients that could influence keloid recurrence. Patients with increased likelihood of keloid recurrence should be identified through proper history and physical examination followed by relevant laboratory tests They should be managed by either non - surgical methods or be given a higher dose of post excision superficial radiotherapy. They could further be monitored closely so as to intervene as soon as any sign of recurrence occurs

CHAPTER ONE: INTRODUCTION

1.1 : Definition

Keloid from a Greek word meaning "crab's claw" is a fibro-proliferative disorder characterized by abnormal deposition of collagen within a wound. This dermal "tumour" spreads beyond the margin of the original wound and continues to grow over time. It commonly recurs following excision and would be present for a minimum of one year. In spite of many treatment modalities, keloids have been shown to have high recurrence rates (Chike-Obi, 2009). Factors that seem to influence keloid recurrence have been poorly understood making it hard for physicians or patients to determine the outcomes of keloid treatment.

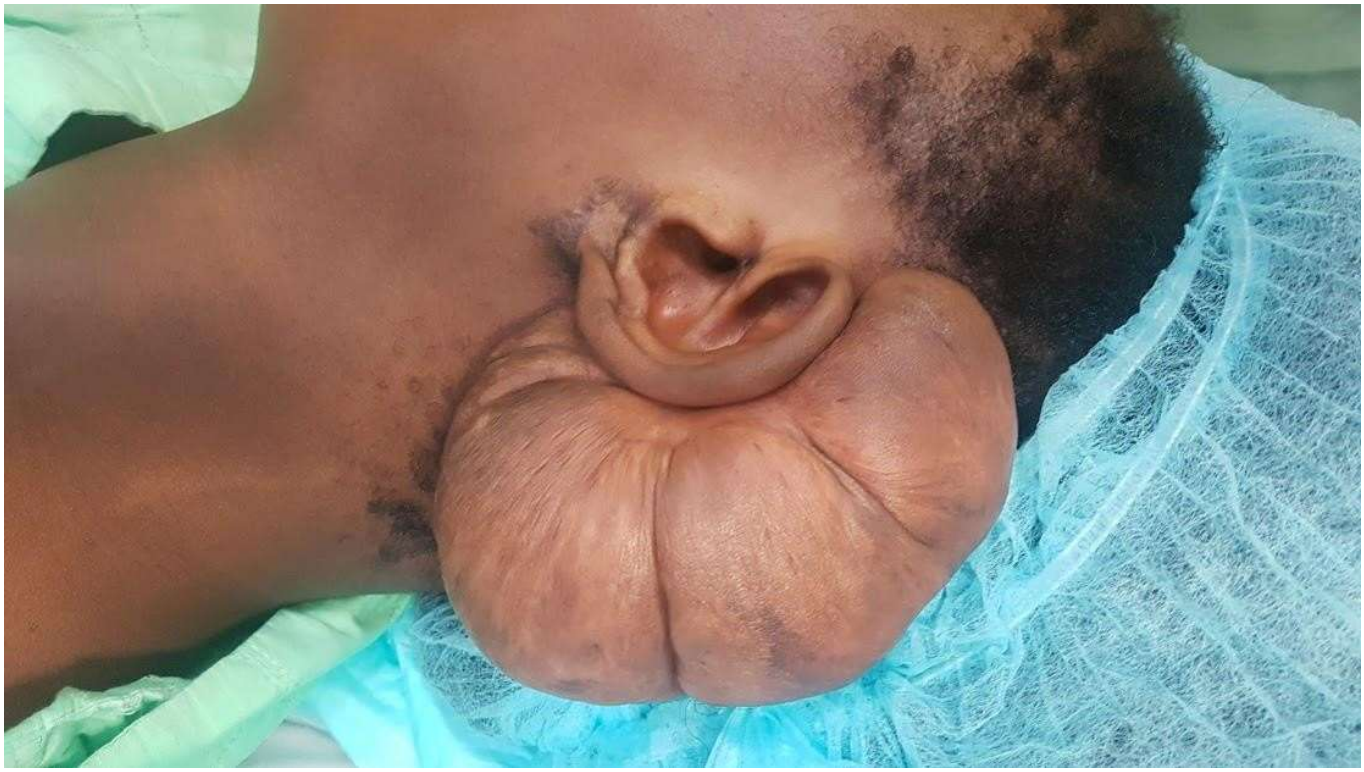

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Figure 1: Patients with large recurrent left auricular keloid

1.2 : History

Keloids were first reported in literature in 2000 BC. The report by Smith Papyrus described it as firm, nodular, large and hard swellings on the breast. In early 1800 BC, the word keloid was coined from the Greek word 'chele' meaning the crab's claw by Alibert. He described keloids as arising both spontaneously and from trauma. He also differentiated keloids from hypertrophic scars.

1.3 : Epidemiology

Keloids have been noted to be more common in certain body parts. These body parts include parasternal areas, earlobes, deltoid and thepost auricular region (Chike-Obi, 2009; Kiprono, 2015). They are less common over the palms of the hand, scrotum, penis and the upper eyelids. The relatively high frequency in females is thought to be due to the ear-piercing practice as well as a more cosmetic conscious female gender as opposed to the male (Chike-Obi, 2009; Davis, 2013). They are more common in Asians and Blacks with an incidence of 5:1 and 15:1 compared to the Caucasians respectively (Brissett, 2001; Ramakrishna, 2014; Kiprono, 2015 and Davis, 2013). Individuals with HLA B14, BW16, DR5 and blood group A are more prone to keloid formation (Chike-Obi, 2009; Shih, 2012).

The local incidence of keloids is not known; however, data from the USA reports a prevalence of 16% among African - Americans (Davis, 2013; Shih, 2012). Davis *et al* (2013) reported that 425,000 visits were done in the USA for treatment of keloids between 1990 and 2009. Majority were female contributing to about 64% with about 23.7% being African – Americans. Keloids were originally thought not to occur in albinos; however, a study done in Kenya by Kiprono *et al* (2015) demonstrated an equal incidence of keloids in albinos and non - albino populations. In

addition, there is increased incidence of keloids in puberty and pregnancy (Shih, 2012). This is attributed to increased levels of melanocyte stimulating hormone (MSH) (Shih, 2012; Jumper, 2015). MSH is thought to increase the tendency of keloid formation by 20 folds (Jumper, 2015).

1.4: Pathogenesis of Keloids

The pathogenesis of keloids is still not clear (Nang'ole, 2019). Theories that seek to explain keloid formation include, tension, hypoxia, abnormal fibro-blasts proliferation, genetic and inflammation (Nang'ole, 2019, Jumper, 2015). It is postulated that Keloid formation is because of a poorly regulated mechanism in wound healing involving fibroblasts, cytokines and metalloproteinase (Abdou, 2011; Jumper, 2015).

Fibro-blasts are the main cells involved in the synthesis of extra-cellular matrix in the body. They deposit collagen fibres responsible for wound healing. Fibro-blast's activities are modulated by cytokines, TGF-β and EGF (Jumper, 2015). The TGF-β inhibits the synthesis of matrix metalloproteinase9 (MMP9), which is responsible for the degradation of the collagen fibres in wounds (Brissett, 2001; Abdou, 2011). Keloid fibro-blasts have been shown to be more sensitive to TGF-β and PDGF that results in excessive synthesis of collagen (Brissett, 2001; Jumper, 2015). In addition, keloid fibro-blasts have been shown to resist apoptosis and continuously proliferate once stimulated by TGF-β (Jumper, 2015).

Histologically, there is increased collagen type 1 as compared to type 3 in keloidal tissues (Ehrlich, 2014; Kischer, 2011; Lee, 2004; Moshref, 2010; Alexander, 2009 and Reno, 2013). Even more important is the haphazard manner by which collagen fibres are arranged as opposed to the linear and regular pattern that is found in normal skin (Ehrlich, 2014; Kischer, 2011; Lee, 2004; Moshref; 2010; Alexander, 2009 and Reno, 2013).

1.5: Management of keloids

There are many modalities currently in use for managing keloids. This is probably a pointer to the fact that there is no ideal or universally accepted treatment modality. Apart from surgical management of keloids, other modalities of treatment include pressure therapy, silicone-based products, cryotherapy, verapamil, triamcinolone acetate, antimitotic agents and radiotherapy (Mustoe, 2008). The effectiveness of these treatment modalities vary widely with none showing consistent results in prevention of keloid recurrence. To reduce on the recurrence, at least two modalities of treatment are advocated for (Mustoe, 2008).

1.6: Research Question

What factors influence keloid recurrence in surgically managed patients at Kenyatta National Hospital?

1.7: Broad objective

To determine the clinical, genetic, inflammatory markers and histopathological variants in keloids as predictors of recurrence

1.8: Specific Objectives

1. To assess whether the clinical presentation and surgical management of keloids is a determinant to keloid recurrence.
2. To determine the HLA sub-types and blood groups in patients with keloids and their correlation to recurrence.
3. To determine the association between inflammatory cytokines and keloids recurrence.

4. To determine the gross histological / immune-histochemical composition of keloids and the relation to recurrence.
5. To determine the mesenchymal stem cells in keloid specimens and any association to recurrence.

1.9 : Null hypothesis

There is no correlation between the clinical, surgical, genetic, inflammatory cytokines and histological composition of keloidal tissue and recurrence.

1.10 : Study justification

Keloids are common pathological conditions among African patients. Management of keloids is difficult and recurrence after treatment is often common (Chike-Obi, 2009 and Gold, 2020). A higher recurrence rate leads to a patient's dissatisfaction and psychosocial trauma. No studies had been done to determine factors that influence keloid recurrence among African patients both locally and internationally. Keloid disease has been shown to have a strong genetic predilection with a strong familial tendency. Whether this could influence recurrence among our patients had not been documented. The histological composition of keloid tissue is heterogeneous. Among the differences, include inflammatory cells such as the keloid associated lymphoid tissues, mesenchymal stem cells and fibro-blasts densities. There was no study done to correlate the clinical and histological composition of keloidal tissue as a determinant to their recurrence in our setting. Previous studies had documented patients with certain blood groups and HLA sub-types to be more prone to keloid formation. However, no correlation had been done as far as keloid recurrence was concerned. This study might be able to influence management of patients with keloids by having a preformed mind on the possibilities of recurrence both to the physicians and

to the patient prior to surgery. The study might also open up new avenues of drug interventions in the treatment of keloids. Biomarkers on keloid severity and recurrence might be developed in future based on this study.

CHAPTER TWO: LITERATURE REVIEW

2.1 : Keloid Recurrence

Difficulties in understanding the extent of keloid recurrence stems from the fact that there is no universally agreed definition. Majority of the published data define recurrence in terms of keloid re-growth with no mention of other clinical features. Keloid recurrence is however, best defined in terms of the entire clinical spectrum of re-growth, pain and pruritus, which are of most concern to patients (Gold, 2020). Factors that have been quoted in literature that could influence recurrence are patient's sex, anatomical location, size of the keloids, infection and family history of the disease (Park, 2015; Sun, 2017; Micheal, 2020). Park *et al* (2015) in a retrospective review of paediatric patients managed by surgical excision and post excision steroid injection, noted recurrence to be higher on the chest and the lower limbs. Sun *et al* (2017) in another study noted keloid recurrence to be more common in areas of high skin tension such as the chest compared to low skin tension such as the ears. He however noted that patients' age, sex, family history of keloids and surgical method did not influence keloid recurrence. Shin, (2016) in a comparative study between post-surgical triamcinolone and radiotherapy found no significant difference in the recurrence of the auricular keloids. He found male gender as the main prognostic factor with male patients having a recurrence of 45.3% compared to the female patients with a recurrence rate of only 9.9%. Higher recurrence was also noted in patients with infected keloids and with family history of keloids (Michael, 2020).

Majority of the above studies are however retrospective leading to a low level of evidence. They further define keloid recurrence in terms of keloid re-growth with no consideration of the clinical

features of pruritus and pain, which are common features in keloid recurrence. In addition, none of these studies was done in keloid endemic areas such as sub-Saharan Africa.

2.2 Clinical Presentation of Keloids and recurrence

Most keloids develop in early adolescence and before the fourth decade of life (Gold, 2020). Although uncommon in the paediatric age, severe keloids have been reported in very young patients especially after burn injuries (Gold, 2020). Another common cause of early childhood keloids has been chicken pox infections (Jumper, 2015). The tendency for recurrence has not been documented in literature, whether it correlates with the age of presentation needs to be established.

Common clinical features in patients with keloids are pruritus and pain. Lee *et al.* (2004) in a retrospective review of 28 patients noted pruritus as the most common presenting complaints. He observed pruritus in 86% of the patients while 46% reported pain. Pruritus is thought to be driven by mast cells that release histamine in keloid patients. Pruritus severity is thought to correlate with mast cell density in the keloid specimens. Many studies on keloid recurrence have however not determined whether pruritus or pain severity could correlate with recurrence.

Majority of keloids have been noted to be in the head, neck and chest areas of the body (Bagabir, 2012). Ramakrishnan,*et.al* (2014) in retrospective review of 1000 keloid patients found 34% of the patients with keloids in the parasternal area, 17% deltoid, 13% upper limb and 9% in the ears. Interestingly, histological studies on cadaveric skin from various parts of the body have shown skin from keloid prone areas to have a high density of collagen and a low macrophage count compared to non - keloid prone sites (Butzeelar, 2017). It has also been postulated that keloids prevalence is higher in these regions because of the high skin tension (Chike-Obi, 2009; Jumper,

2015; and Bagabir, 2012). Keloid recurrence has also been shown to be higher in some areas of high skin tension such as the chest, thus re -emphasizing the role of skin tension in keloid formation and recurrence (Sun *et al*, 2017). Ironically, however, ear lobes though a common site for keloid formation, do not have much skin tension. The lower extremities on the other hand with a lot of skin tension do not form as much keloids as the upper limb and the chest region implying that tension alone could not explain keloid pathogenesis and recurrence.

2.2.1 : Surgical excision of Keloids and recurrence

There has been no consensus on the optimal surgical excision technique for keloids (Gold, 2020). Surgical excision of keloids has been either by intra-lesional or extra-lesional technique**.** While intra-lesional excisions of the keloid leaves a rim of keloid tissue, extra-lesional excisions result in complete excision of the keloid tissues, leaving a wound that is fully devoid of keloids. Proponents of intra-lesional excisions argue that keloid disease is stimulated by trauma to normal skin. Therefore, excising keloids out of their margins may stimulate normal skin into keloid formation, resulting in higher recurrence rates (Moshref, 2010). The argument is supported by the fact that patients with keloids tend to have them anywhere else on the body once stimulated by any form of trauma. They further argue that the histological active part of the keloid is the central core, and removing it leaves a relatively inactive peripheral zone, reducing the chances of further keloid overgrowth (Moshref, 2010). However, available evidence suggests otherwise. Syed *et al*. (2011) demonstrated that fibro-blasts from the growing margins of keloids produced more collagen than fibro-blasts from intra-lesional or extra-lesional sites. Further, Reno *et al.* (2013) in an *in vitro* study found that fibro-blasts from different parts of keloids demonstrated similar doubling times and did not show central fibro-blasts to be more active than those from other sites. Extra-lesional excision of keloids is based on the assumption that keloids should be treated as a form of 'neoplasm' and should thus be managed with wide local excision based on the oncological principles of neoplasm management. The assumption in this school of thought is that keloid-forming tissue has a proliferating disease core, as suggested by Chong *et al*. (2018) that transforms into the keloid and must thus be fully removed. This raises the fundamental

question of whether keloid formation is an excessive reaction to body injury or a case of abnormal fibro-blast proliferation. *In vitro* studies have demonstrated that normal fibro-blasts may be stimulated into keloid formation by cytokines from keloid fibro-blasts, putting doubt to the proliferating disease core concept (Nast, 2012). Clinical experience has also shown that keloid-forming patients have a tendency to form keloids virtually anywhere on the body after any form of trauma, suggesting that keloid disease is not a local but a regional or `systemic disease. There is however little evidence that either method of excision has more recurrences (Chong, 2018; Gold, 2020and Tan, 2010).

2.2.2 : Histological composition of keloids and recurrence

The role of keloid histology in determining keloid severity and recurrence has not been fully established. Keloids tissue has been shown to be heterogeneous in composition with varying amounts of inflammatory cells, fibro-blasts and capillary densities (Ali, 2020; Jumper, 2015). Whether these variables correlate with keloid severity and recurrence is not known.

Keloids are thought to form as a result of abnormal wound healing due to an exaggerated inflammatory and proliferative phase (Brissett, 2001;Nangole,2019). Due to a prolonged inflammatory phase, there is a sustained release of cytokines and growth factors, which in turn stimulate fibro-blasts to proliferate and deposit excessive extra-cellular matrix (Brissett, 2001). Experimental studies have demonstrated that a prolonged inflammatory period with immune cell infiltrate increases fibro-blast activities that lead to a more sustained extra-cellular matrix formation akin to keloid formation (Jumper, 2015). This finding has been supported by histological findings, which have demonstrated the presence of inflammatory infiltrates in many keloid tissues (Jumper, 2015; Boyce, 2001; Martin, 2009 and Shaker, 2011).

However, not all keloid specimens have demonstrated a high concentration of these cells. The densities of these cells also vary from keloid to keloid even in the same individual. The inflammatory response has further been noted not to be uniform in the entire keloid (Shaker, 2011). The significance of these variations in keloid severity and recurrence has not been demonstrated. Park *et al*. (2016) on investigating the role of macrophages and giant cells in the keloid specimens on recurrence concluded that there was no evidence to demonstrate that keloid specimens with Langerhans cells had a higher recurrence than those without. Their study was however limited by a small sample size with only seven patients having keloids with foreign body reaction. In another study by Park *et al.* (2015), again, on whether histo-pathological parameters affect the rate of recurrence in auricular keloids, demonstrated that that was not the case. The parameters analyzed in the study were gross morphology, margin involvement, lymphocytic infiltration and keloidal collagen area. Others were presence of epidermal cysts and foamy histiocytes. Key cells not analyzed in this study were the presence or absence of the mast cells.

Lymphocytes shown to be commonly increased in the keloid tissue are the T-lymphocytes (Brissett, 2001; Thomton, 2021 and Gold, 2020). Martin and Muir (2009) demonstrated not only an increase in the T-lymphocytes but also a high CD4: CD8 ratio. He also demonstrated an aggregation of lymphoid tissue in the keloid referred to as the keloid associated lymphoid tissues in at least 15% of the histological specimens. Kim *et al.* (2012) showed that memory T-cells were the predominant T-cells in the Keloid tissue and were probably responsible for the abnormal inflammatory response. Chen *et al*. (2018) on the other hand found keloid tissues to have elevated T-regulatory cells compared to the normal skin. While lymphocytic infiltration in

most malignant tissue is associated with good prognosis, it is not clear what influence T-lymphocytes could have on the severity and recurrence of keloids.

Mast cells have been found to be high in most keloid tissues (Tracia, 2014; Smith, 2007; Ong, 2010 and Wang, 2011). Their role in the causation and recurrence of keloids is unclear. There is however growing evidence that mast cells play a critical role in wound healing and abnormal scar formation (Tracia, 2014; Smith, 2007; Ong, 2010 and Wang, 2011). In wound healing among the mice models, scarless wound healing was observed in mice foetus with no mast cells at the embryonic age of 15 days as opposed to scar healing in mice of the embryonic age of 18 days with mast cells bringing to the fore the critical role they could play in scars, keloid formation and recurrence (Wilgus, 2020). Brissett (2001) and Shaker, (2011) showed mast cells to be in close contact with fibro-blasts suggesting a paracrine activity. Mast cells have also been thought to produce cytokines and other factors such as histamine that seem to stimulate fibro-blast activities leading to collagen deposition and scar formation (Martin, 2009). *In vitro* studies with mast cells and fibro-blasts found mast cells to increase proliferation of the fibro-blasts and increase collagen synthesis, similar to what happens in keloids suggesting that they could play a critical role in keloid synthesis and probably recurrence (Santra, 2015; Wilgus, 2020).

2.3 : Stem Cells and Keloid Recurrence.

Stem cells, pluripotent cells with ability to differentiate into other cells have been shown to play a critical role in wound healing. Their role however in keloid pathogenesis, treatment and recurrence is not clear (Kilmister, 2019). This is inspite of the fact that Keloid tissues have been demonstrated to have a high concentration of stem cells than normal skin (Akino, 2008).

Stem cells are thought to provide keloids with a steady supply of cells that differentiate into myofibro-blasts and fibro-blasts once triggered by the pro-inflammatory cytokines such as IL-6 and IL-17and thus ensure a continuous and steady synthesis of collagen resulting in keloid formation (Kilmister, 2019). Further, embryonal-like stem cells in keloid-associated tissues do have Vitamin D receptors suggesting that either an interaction or lack of it between Vitamin D and these cells could play a role in keloid pathogenesis (Brissett, 2001; Qunzhou, 2009; Kilmister, 2019).These cells also seem to be stimulated through autocrine activities by keloid fibro-blasts (Brissett, 2001 Akino, 2008 and Akino *et al.* (2008).

While several authors have demonstrated mesenchymal stem cells to be the predominant stem cells in the keloid specimens (Qunzhou, 2009; Kilmister, 2019), haematopoietic stem cells have also been noted by other authors like Kim (2012) andBakry(2014). Bakry *et al.* (2014) in their study on the comparative analysis between keloid specimens and normal skin biopsy demonstrated haematopoietic stem cells to be more marked in keloid specimens than in the normal skin by about 76%. Iqbal *et al.* (2010), on the other hand, demonstrated that both haematopoietic and mesenchymal stem cells were common in keloid specimens with mesenchymal stem cells more in the core areas of the keloids while haematopoietic stem cells were more in the periphery of the keloids. The significance of these findings in the pathogenesis and recurrence of keloids is still unknown. Ironically, more and more studies seem to demonstrate that stem cells may be utilized in the treatment of keloids and hypertrophic scars (Spiekmann, 2014; Qianku, 2016; Zahorec,2021; Liu, 2017 and Bommie,2016). Spiekman *et al.* (2014) showed that adipose tissue-derived stromal stem cells inhibit TGF-beta1-induced differentiation of human dermal fibro-blasts and keloid scar-derived fibro-blasts in a paracrine fashion. Bommie (2016) demonstrated mesenchymal stem cells to have a role in the management

of hypertrophic scars and probably keloids. Fong (2014) in the *in vitro* culture of keloid fibro-blasts with Wharton's derived stem cells demonstrated a significant reduction of the fibro-blasts activities. This effect is thought to be as a result of the anti-inflammatory cytokines released by the stem cells postulating a possible role of stem cells in the treatment of keloids. A similar study by Ya *et al.* (2017), investigating foetal foreskin derived MSC and keloid fibro-blasts, demonstrated a reduction in keloid fibro-blast activities and eventually resulting in apoptosis of the fibro-blasts. The anti-inflammatory cytokines produced by the MSC include interleukin 10, Nitric oxide and PGE2 (Kilmister, 2019).

2.4: Genetic composition and keloid disease

The concept of genetic predisposition to keloids has been supported by the fact that patients with keloids often report a positive family history (Spiekman, 2014; Bommie, 2016; Fong, 2014; Ya, 2017; Bayat, 2013 and Marneros, 2011). Keloids have also been found to have an equal sex distribution, suggesting an autosomal mode of inheritance. Keloid formation is observed in all races but dark-skinned individuals have been found to be more susceptible to keloid formation with an incidence of 6 to 16% in African populations (Davis, 2013; Shih, 2012). Further, the incidence of keloids in Africans and albinos of African descent has been reported to be similar, implying a strong genetic predisposition. Keloid recurrence has however been shown by some studies to be more common in male than female patients. Genetic studies on families with keloids have suggested an autosomal dominant mode of inheritance. Marneros *et al* (2014) studied two families with keloids and found an autosomal mode of inheritance. He identified linkage to chromosome 7p11 and chromosome 2q23 for the African and Japanese family, respectively. Brown *et al* (2008) found genetic association between HLA-DRB1*15 and the risk

of developing keloids in white individuals. Also, carriers of HLA-DQA1*0104, DQB1*0501 and DQB1*0503 have been reported to have an increased risk of developing keloid scarring (Jagajeevan, 2007). No studies have however been done to demonstrate whether there is any significant relation between these alleles and keloid recurrence.

2.5: Cytokines, Growth Factors and Keloid Disease

Cytokines and growth factors implicated in keloid formations include the transforming growth factors, matrix metalloproteinase and interleukins (Marneros, 2014; Bayat, 2004 and Brown, 2008). Transforming growth factors are released from the platelet granules during the process of inflammation. TGF-β1and TGF-β2 are thought to promote scar formation and fibrosis while TGF-β3 are thought to reduce scar tissue (Brissett, 2001; Jumper, 2015; Oliver, 2005). TGF-β1 and B2 are thought to work by down- regulating the effects of MMPS, especially MMP9 and thus result in reduced collagen degradation and hence abundance of collagen in the tissues (Brissett, 2001). Keloid fibro-blasts have been shown to be very sensitive to TGF-β_1 causing an increase in absolute collagen synthesis (Brissett, 2001; Jumper, 2015). Patients with keloids have also been shown to have high levels of the TGF-β1 in circulation (Brissett, 2001). Anti-bodies against TGF-β in animal models have been shown to reduce the effects of keloid fibro-blasts resulting in reduction of fibro-blasts activities (Ying, 2016).

Interleukins associated with keloid formation have predominantly been the inflammatory type (Wilgus, 2020). These include IL-6 which has not only been shown to be elevated in patients with keloids but also in acute and chronic inflammatory condition such as rheumatoid arthritis and systemic lupus erythematosus (Kilmister, 2019; Azzza, 2019; Tosa, 2016). IL-6 plays a critical role in both cellular and humoral immune response and has been suggested as a possible

target for keloid treatment and prevention (Uitto, 2007). It stimulates chronic inflammatory response by recruiting monocytes into the wounds (Kilmister, 2019). IL-6 also regulates stem cells function via several pathways leading to the inhibition of apoptosis and maintenance of haematopoietic stem cells (Akino, 2008). It is thought to play a role in malignant transformation as established during the transformation of colitis to colonic cancer (Bayat, 2004). Its effects are thought to be amplified by IL-17 (Marneros, 2011). Quanzhou, *et al* (2009) demonstrated IL-6 to be greatly increased in keloids than in normal skin. They further demonstrated the ability to produce keloid-like tissue in immune-compromised rats by using keloid derived stem cells and IL-6. These activities were stopped by using anti-bodies against IL-6. McCauley, *et al.* (2010) demonstrated monocytes of patients who form keloids to produce large amounts of IL-6, TNF-α and IFN-β compared to the monocytes of the normal control populations. Hui Xue *et al.* (2000) demonstrated an elevation in the expression of the IL-6 genes in the keloid fibro-blasts as compared to the normal fibro-blasts. X.J. Zhu, *et al* (2017) recently demonstrated IL-6 to be in high concentration in patients who form keloids than in the normal population. D.V. Do *et, al.* (2012) also demonstrated IL-18 to be involved in the pathogenesis of keloids. No studies have been done to determine any correlation between these cytokines and keloid recurrence.

CHAPTER THREE: METHODOLOGY

3.1*: Study Design*: Case Control observational study.

***Setting*:** Plastic Surgery Clinic, Kenyatta N Hospital and KAVI Institute of clinical research (University of Nairobi)

***Study Population*:** Patients with keloids presenting at the plastic surgery clinic, Kenyatta National Hospital

Study Duration: August 2018- August 2021

Sample Size Determination and Sampling Procedure

Sample Size Determination

The study sample size was calculated by using two -arm non-inferiority sample size formula as cited by Liu *et al.* (2018) [HYPERLINK \l "Jun14" 1] and Azmee *et al* Liu 2018)

$$n_t = \left(\frac{z_{1-\beta} + z_{1-\alpha}}{\varphi(\pi)}\right)^2 \left[\pi_t(1-\pi_t) + \frac{\theta^2\pi_c(1-\pi_c)}{C_c} + \frac{(1-\theta)^2\pi_p(1-\pi_p)}{C_p}\right]$$

Where $N = n_t + n_c + n_p = n_t\,(1 + C_c + C_p)$

$$\varphi(\pi) = \pi_t - \theta\pi_c - (1-\theta)\pi_p$$

$n_c$is the sample size for intralesional keloid group.

$n_p$is the sample size for extralesional group group.

In this study, $n_t = n_c + n_p$ therefore $C_c = C_p = 1$

θIs the non-inferiority margin, which according to Koch and Tangen [HYPERLINK \l "Koc99" 3] the reasonable region for non-inferiority test is for θ between 0.5 and 0.99 for this study θ is set at 0.5.

π_pIs the recurrence rate for control group (0%).

π_cIs the recurrence rate for intra-lesional surgery and radiotherapy arm that is 20% the work has been reported in line with the STROCSS criteria

π_tIs he recurrence rate for extra-lesional surgery and radiotherapy that is 20%.

$z_{1-\beta}$ Represents the desired power (0.84 for 80% adopted for this study).

$z_{1-\alpha}$ Represents the desired level of statistical significance (1.96 for 95% adopted for this study)

Therefore, applying the above, we get

$$\varphi(\pi) = 0.295 - 0.5 \times 0.20 - (1 - 0.5) \times 0 = 0.195$$

$$n_t = \left(\frac{0.84 + 1.96}{0.195}\right)^2 \left[0.295(1 - 0.295) + \frac{0.5^2 \times 0.20(1 - 0.20)}{1} + \frac{(1 - 0.5)^2 \times 0 \times (1 - 0)}{1}\right] = 84$$

Therefore, 84 patients were recruited for each arm translating to 168 patients with keloids. However, on average between 100 and 200 surgeries are cases of keloids at KNH. Applying the finite correction factor:

$$n = \frac{n_0}{1 + \frac{n_0 - 1}{N}} = \frac{168}{1 + \frac{168 - 1}{150}} = 79.5 \approx 80$$

A minimum sample size of 80 patients with keloids in KNH will be targeted but to cater for loss on follow up an additional 10% will be factored in the final sample size translating to 88 patients with keloids (44 for each arm) and 44 healthy persons.

3.2 : Sampling Procedure

The researcher kept a consecutive list of patients undergoing keloids management at KNH throughout the study period, which composed the study-sampling frame. Prior to the day of keloids management, the researcher obtained the list of patients scheduled to undergo keloids management for the next day. Systematic random sampling was applied on a rolling basis to randomly sample study subjects from study sampling frame whereby every second patient scheduled to undergo keloids management by surgical intervention at KNH was recruited for the study.

3.3 : Randomization

The study participants were randomized via block randomization to reduce bias and achieve balance in allocating participants to the treatment arms. The treatment arms were intra-lesional excision plus post excision superficial radiotherapy and extra-lesional excision plus post excision superficial radiotherapy (see flow chart below, figure 2). Within each block, however, the order of patients was random (This study utilized blocks of size 4 with allocation ratio of two intra-lesional surgery and radiotherapy: two extra-lesional surgery and radiotherapy). Within each block, patients were assigned random numbers generated by Excel and the random numbers assigned were used to order the patients within the block. Since there were six different ways of assigning treatments in blocks, the blocks received a treatment method by lottery method whereby two uniform pieces of papers each represented a treatment plan. For each block, the researcher randomly picked the piece of paper and used it as the treatment plan for the patient.

3.4 : Selection criteria

3.4.1 : Inclusion criteria

1. Patients with Keloids who consented to the study.
2. Patients with keloids amenable to surgery with possibilities of primary wound closure.

3.4.2 : Exclusion criteria

1. Patients with keloids who had had surgery within a year for keloid.
2. Patients with extensive keloids that could not be closed primarily.
3. Patients with clinically infected keloids
4. Patients with chronic medical conditions

3.5 : Study limitations

1. Biasness in the analysis of histological specimens
2. Keloids cellular composition is not homogenous
3. Subjectivity in the clinical assessments of keloid symptoms.

3.6 Study delimitation

1. Experienced dermato-pathologist analyzed all the gross histology specimens
2. Multiple samples in each keloid, a minimum of two were assessed to give a better presentation of each tissue from the different anatomical locations of the keloid.
3. Hot spot technique was utilized to analyze keloid histology
4. Pain and pruritus visual analogue scales were utilized to assess the clinical features in all patients

3.7 : Procedure

3.7.1 : Clinical presentation and surgical management

Patients with keloids seen at the plastic surgery clinic at Kenyatta National Hospital who met the inclusion criteria and assented to the study were recruited. A detailed history was taken to determine the onset of keloids and the causative agents. Family history was taken to determine the presence or absence of any members with keloids. A clinical examination was then done to determine the extent, number and location of keloids. The keloid surface area was measured using the grid iron technique. Pruritus and pain were assessed by the pruritus and pain visual analogue scales, respectively (See Appendix 2 and 3). Patients were randomized into two groups using a computer-generated programme as discussed above. The groups were then managed as follows:

Group A: Intra-lesional surgical excision plus post excision superficial radiotherapy

Group B: Extra-lesional excision plus post excision superficial radiotherapy

All surgeries were done by the principal investigator or by one of the senior plastic surgeons in the department of surgery at KNH. The surgical technique was by either intra-lesional (IL) or extra-lesional excision (EL) of keloids followed by primary wound closure. Intra-lesional excision involved cutting keloids with at least 3mm of keloid tissue left behind while extra-lesional involved cutting keloid tissue with at least 3 mm of normal skin (Figures 5 -8). Wounds were then closed in two layers with dermal stitch using polyglycolic acid (vicryl) 3/0 and trans-cutaneous stitch with nylon 3/0 interrupted sutures. All patients were subjected to 15 grays of external beam radiotherapy within 24 hours of excision. The principal investigator reviewed post-surgery patients at one week, one month, three months, six months, and at least at one year. At each review, keloid scar surface area, pain and pruritus scores were taken (See Appendix 1). Surgical or radiotherapy related complications were also documented.

Keloid recurrence was determined as either,

1. Any scar that healed beyond the margins of the initial scar at least at one year of follow up as determined by the surface area of the scar tissue.
2. Pruritus or pain scores that worsened in the course of follow up.
3. Pruritus or pains scores that necessitated further medical interventions to control in the course of follow up

3.7.2 : Histology /Immuno-histochemical/ Stem cell Analysis

The excised tissues were divided into two portions. The first portion was taken to the Department of Histo-Pathology, University of Nairobi for histology. The second portion was taken to KAVI laboratory for quantification of the stem cells. All histology specimens were fixed with

paraformaldehyde, processed and stained with H and E, Masson and Trichome stains. The processed slides were analyzed for the cellular components and extra-cellular matrix density using high power magnification of light microscopy. The cells analyzed were lymphocytes, macrophages, mast cells and fibro-blasts. An absolute count for these cells was noted in each slide with an average made from the periphery and central part of the keloid. The extra-cellular matrix was analyzed for blood vessels density and the collagen fibres (See Appendix 1).

Within the KAVI laboratory, the second portion was analyzed for the mesenchymal stem cell (MSC) densities using flow cytometry (See appendix 6). MSC were characterized by the expression of stromal cell markers (CD73, CD105, CD44, CD29 and CD90) in the absence of hematopoietic markers (CD34, CD45 and CD14) and endothelial markers (CD34, CD31 and VWF). The absolute MSC count per specimen was divided by the weight to get the density of MSC per gram of tissues. In each specimen, MSC percentage was analyzed from the total cells count. Immuno-histochemical staining was done on selected slides and stained for CD68 and CD163 antigens for the macrophages (See Appendix 8). The mast cells were assessed using anti-tryptase and anti-chymase antibodies (Mario, 2013; Monika, 2016; Craig, 2012 and Abe, 2008).

3.7.3 : **Blood groups, HLA sub-types and Cytokines**

Patients' blood was taken using sodium heparin specimen bottles for analysis of the blood groups, HLA sub-types and inflammatory cytokines. HLA typing was done using sequence specific primer genotyping (SSP). DNA extraction was done using Qiapen extraction kitTM. PCR master mix preparation was done by adding commercial kit from OlerrupTM. PCR master Mix was aliquoted into 96 well plates containing Primer Mixes. PCR amplification was done

using QiaxelTM automated machine, which uses a commercial cartridge. Interpretation was done by SSP-typing HLA allele software (SeeAppendix 5).

Cytokines assay were done using Bio-plexmultiplexElisa^{TM} assay technique manufactured by Bio –Rad Company based at Berkeley, California, USA. The technique involved use of fluorescent dyed micro-phores (beads) each with a distinct colour code to permit identification of the individual cytokines. Within a multiplex suspension, coupled beads reacted with the sample containing the cytokines of interest. After a series of washes to remove unbound protein, abiotinylated detection antibody was added to create a sandwich complex. The final detection complex was formed with the addition of streptavidin-phycoerythrin (SA-PE) conjugate. Phycoerythrin served as a fluorescent indicator analyzed by Bio-Plex Data Pro, which presented data as median fluorescence intensity (MFI) as well as in concentration (pg/ml). The concentration of analyte bound to each bead was proportional to the MFI of areporter signal (See Appendix 7).

3.7.4 : Control group

Patients undergoing either aesthetic or elective surgical procedures with no history of keloids or family history of keloids were recruited as a control group. This was to assist in determining the baseline levels of blood groups, cytokines, MSC and HLA sub-types in the normal healthy population with no keloids. These patients were matched for age, sex and BMI to the patients who had keloids. They were also followed up for at least one year to determine whether they would form keloids or not. Skin samples at the incision site were taken and analyzed for histology, Immunohistochemistry and mesenchymal stem cell densities as described above. Blood from the control group was also taken and analyzed for HLA sub-types, blood groups and cytokines using the procedures described above.

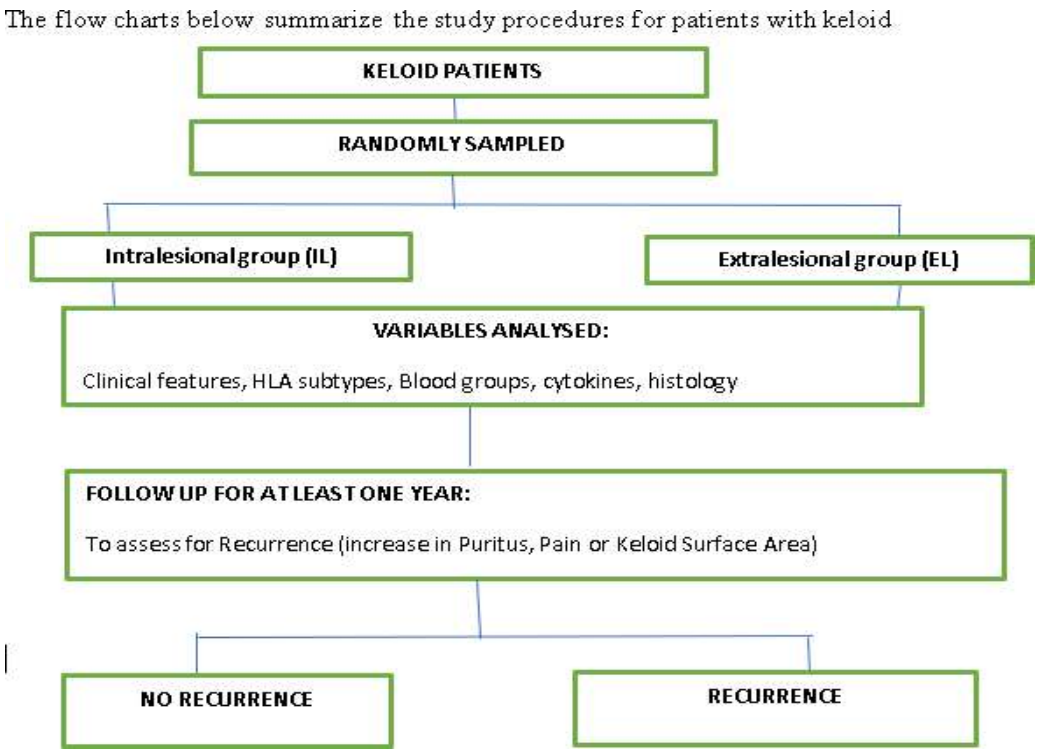

Figure 2: Flow Chart Summary of the study of the patients with Keloids

3.8: Data Collection and Analysis

Data were collected using pre-designed questionnaires (See Appendix 3), then cleaned, coded and analyzed using Statistical Package for Social Sciences (SPSS) computer package version 22. Bivariance analysis was done using student T-test to compare means and a univariate analysis by Chi-square. Pearson's correlation co-efficient (r) was used to determine the significance in the relations between the intra-lesional and extra-lesional groups as well as the similarities between the control and the keloids group. Chi-square test was done to compare keloids aetiology, blood groups, HLA sub-types, cytokines and histological parameters between patients who had keloid recurrence and those with no recurrence. The student T-test was used to compare the means of age of presentation, age of onset, keloid surface area, pain and pruritus scores in the two arms of

study. Other variables measured with the student T-test were the mean mesenchymal stem cells and cytokines in the control and the keloids groups.

Multivariate analysis between histological composition, cytokines, blood groups and anatomical location of keloids between the study groups were done using the ANOVA test for variance. *P* - values of <0.05 were considered statistically significant and 95% CI was used to show any significant associations. Bonferreni correction was done to determine the significance of differences between HLA sub-types and keloid formation and recurrence. Multiple regression analysis was done to determine the strength of relations between family history, gender, anatomical location, blood group, keloid aetiology and recurrence. Analyzed data were presented in form of graphs, pie charts, histograms and tables.

3.9: Logistical and Ethical Considerations

Skin biopsy specimens were obtained in accordance with the Institutional Review Board. Approval for the study was got from the Kenyatta National Hospital / University of Nairobi, Ethics and Research Committee (KNH-UoN ERC)(P29/04/2018). A written study explanation to each patient / guardian was provided and an informed consent obtained. The participants had a right to decline being part of the study with assurance of no penalty for refusal to participate. The same standard of care was provided to both participants and non-participants. Confidentiality reigned supreme with respect to information gathered from each participant and there was no additional cost or incentive for participating in the study.

CHAPTER FOUR: RESULTS

4.1 : Patients Demographics

A total of 96 patients with keloids and a control group of 59 patients were recruited for the study. Six patients with keloids did not complete the study leaving 90 patients who were followed up to the completion of the study. Of the 90 patients, each group, IL and EL had 45 patients each. The male to female ratio for the keloid patients was 1:2. For the control group, the male to female ratio was 1:2 with 39 female and 20 male patients. The age ranges for the keloid patients were 15 to 65 years with a mean age of 29.5 and a median age group of 20-25 years. The age range for control patients was 15.5 to 64 years with mean age of 29.7 years and a median age group of 20-25 years. The modal age group of presentation for male patients was 25-30 years while for the female was 20-25 years. There was no statistically significant difference between the keloids group and the control population. Pearson correlation score between patients with keloids and control group showed a strong positive correlation of r = 0.9998.

Age of presentation (years)	Keloid group	Control group	P –value (chi square test)
10-15	3	2	0.776
15-20	19	10	0.018
20-25	18	14	0.264
25-30	15	12	0.398
30-35	12	8	0.264
35-40	10	6	0.264
40-45	7	4	0.398
45-50	4	3	0.776
> 50	2	2	1
Total	90	59	

Table 1: Age of presentation for the two groups of patients

Table1shows that there was no statistical significance differences in the two groups where the Pearson correlation test r = 0.9998

4.2 : Keloids Aetiology and recurrence

The aetiological cause of keloids was as follows: spontaneous 16.6% (n=17), infective causes 20% (n=21) and post traumatic 63.4% (n=66). Post-traumatic causes included any form of trauma, for example, surgical trauma, assault, burns, ear piercing and motor vehicle accidents. Infective causes included acne folliculitis, chicken pox, cellulitis, pyomysoitis and post immunization.

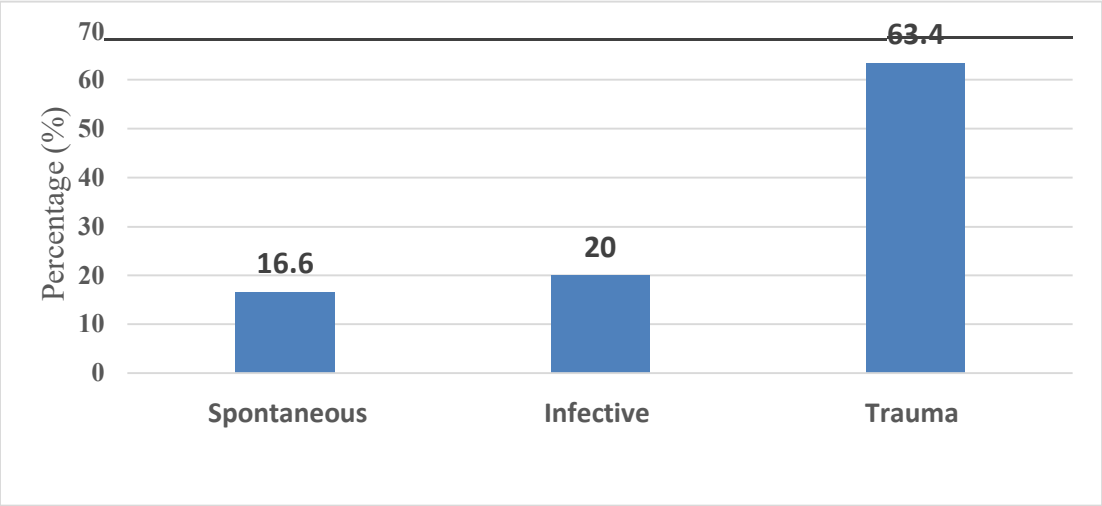

Figure 3: Aetiological cause of Keloids: More than 60 percent of keloids were due to trauma.

A comparison between aetiological causes and keloid recurrence demonstrated spontaneous keloids to have a higher recurrence rate at 29.2 % compared to infective causes at 14% while post-traumatic causes were at 18 %. The difference was statistically significant (*P*-value <0.05).

Aetiological cause	Total (N)	Recurrent (KR) (n) (%)	None recurrent (NKR) (n) (%)	*P*-Value(student T test)
Trauma	66	12 (18.2)	54 (81.8)	<0.001
Infective	21	3 (14.3)	18 (85.7)	<0.001
Spontaneous	17	5 (29.4)	12 (70.6)	0.059

Table2A: Aetiological causes and keloid recurrence. There was significance difference in the recurrence of keloids between the various groups with higher recurrence noted among patients with spontaneous keloids.

Parameters	**Sum of squares**	**Df**	**Mean squares**	**F**	***P*-value**
Between groups	732.05	1	732.05	3.178	0.036
Within groups	921.40	4	115.18		
Total	1653.45	5			

Table 2B: The ANOVA test for Variance demonstrated statistical significance between the various aetiological causes.

4.3 : Clinical presentation and keloid recurrence

Nineteen patients with twenty keloids had keloid recurrence giving an overall patient recurrence rate of 21% and keloid recurrence rate of 19.2 %. The mean age of onset for the patients with no keloid recurrence (NKR) was 24.12 years with an age range of 17 to 65 years compared to the mean age of onset for the patients with keloid recurrence (KR) at 21.70 years with an age range of 15 to 63 years of age(p-value >0.05). The mean age of presentation for the KR was 28.47 years while the NKR was 29.77 years (*p*-value >0.05). The mean surface area of the keloids for the NKR was 9.4 cm^2 with a range from 2cm^2 to 40cm^2 while for the KR the mean surface area was 6.1cm^2 with a range from 1cm^2 to 36cm^2 (*P*-value >0.05). Pain score for the patients with NKR ranged from 0 to 9 with a mean of 1.891 while those with KR the score ranged from 0 to

10 with a mean score of 2.22 (*P*-value>0.05). For pruritus, the range for KR was from 0 to 10 with a mean pruritus score of 4.56 while for the NKR group the range was from 0 to 9 with a mean score of 3.04 (*P*-value>0.05).

In summary, there was no statistically significant difference between the mean age of onset, mean surface area of keloids, pain score and pruritus score between patients who had recurrence and those with no recurrence.

	KR	NKR	*P*-value (T -Test)
Frequency	20	84	
Age of onset	21.70	24.12	0.494
Age of presentation	28.47	29.77	0.712
Surface area	6.1cm2	9.4cm2	0.354
Pain score	2.22	1.891	0.925
Pruritus	4.56	3.04	0.666

Table 3: Mean age of onset, Mean surface area of keloids, Pain score and Pruritus score for keloids with recurrence (KR) and those with no recurrence (NKR). Note there were no statistical significance differences between the two groups.

4.3.1 : Patients gender and keloid recurrence

Of the 104 keloids studied, 38 of them occurred in male patients with 66 in female patients. As seen in Figure 4, male patients had a higher recurrence rate of 31 % compared to female patients at 12 %. The difference was of statistical significance (*p*-value<0.05).

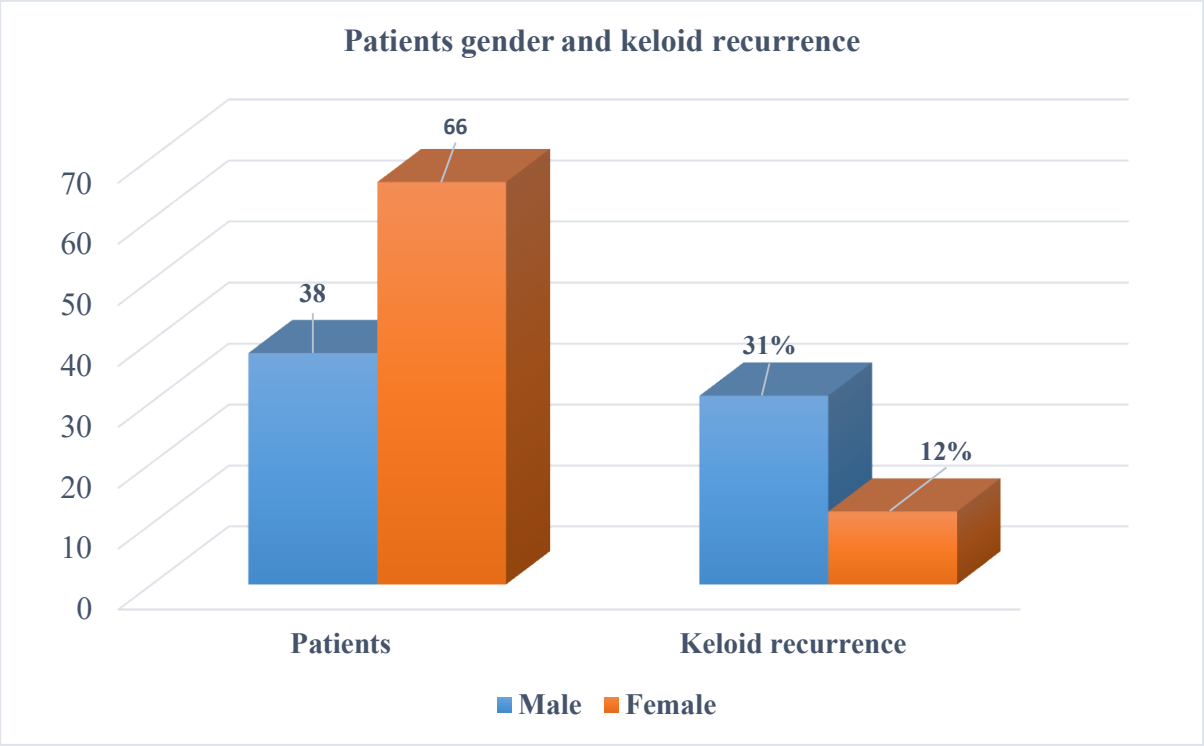

Figure 4: Comparison of patients' gender and keloid recurrence. Male patients had a significantly higher recurrence than female patients at 31 and 12 % respectively (p -value <0.05).

4.3.2 : Familial/Sporadic keloids and recurrence

Forty-seven patients with 54 keloids and who had been operated on had a positive family history of keloids and were classified as familial. The remaining 43 patients with 50 keloids operated on had no known family history and were therefore classified as sporadic. The recurrence rate was significantly higher in the familial group standing at 27.7% compared to 10% in the sporadic group (p-value < 0.026).

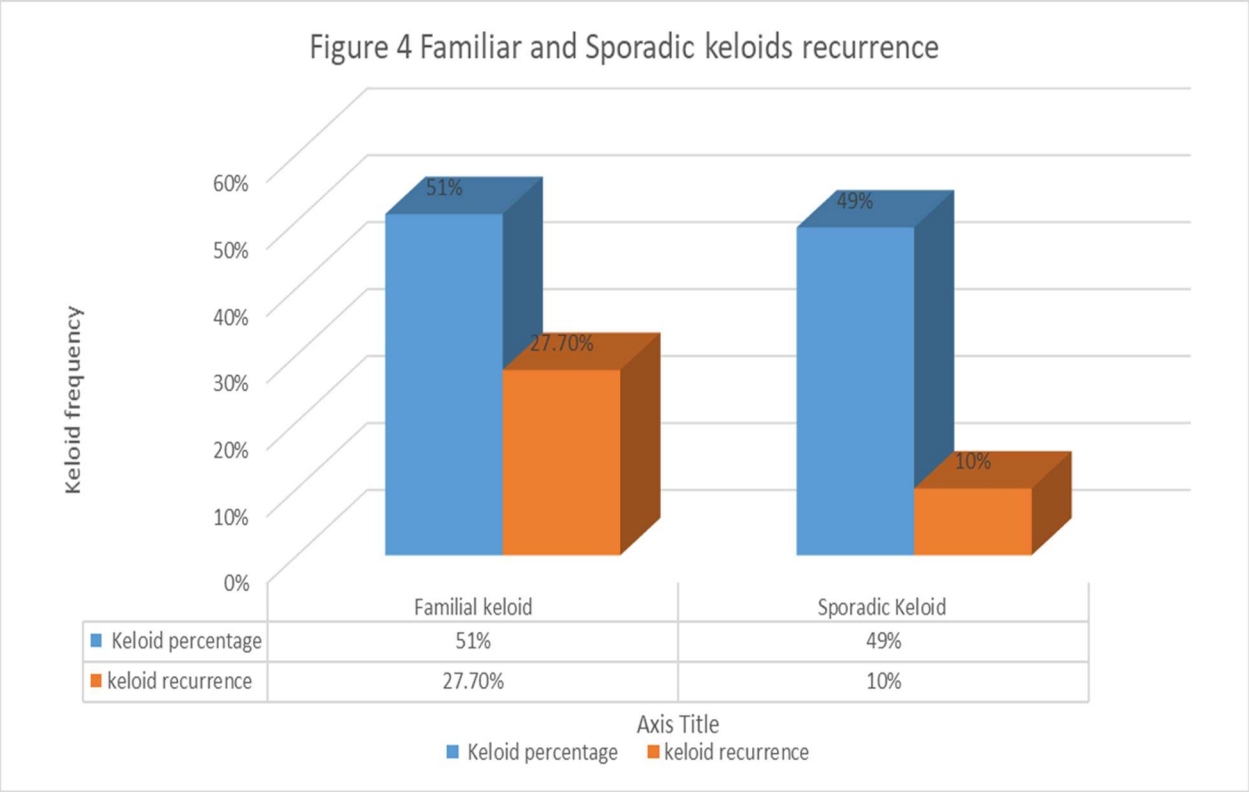

Figure 5: Comparison of keloid recurrence between familial and sporadic group demonstrated that familial keloids had a significantly higher recurrence than sporadic group at 27.7 and 10% respectively (p -value <0.05).

4.3.3 : Anatomical location of keloids and Keloid recurrence

The most common anatomical location of the keloids was the ears accounting for 46% of the keloids with a recurrence rate of 17 .5 % followed by the cheeks at 15% with a recurrence rate of 33%. Others were the chest, back and abdomen with a recurrence rate of 23, 30 and 10% respectively. There was a statistical significance difference between the ears and the cheek, the two most common anatomical locations (P-value <0.05).

Anatomical location	Keloid Frequency N=104	Recurrence N (%)	None recurrent N (%)	*P*-values (Chi square test)
Ears	47	8 (17.1)	39 (82.9)	<0.001
Abdomen	13	1 (7.7)	12 (92.3)	0.005
Chest /Neck	13	3 (23.1)	10 (76.9)	0.059
Cheek	15	5 (33.3)	10 (66.7)	0.167
Upper limb	5	0	5 (100)	0.167
Back	10	3 (30.0)	7 (70.0)	0.264
Scalp	1	0	1 (100)	0.776
Total	104	20 (19.2)	84 (80.8)	<0.001

Table 4 A: Anatomical location of keloids and Keloid recurrence. Higher recurrence rates were noted on keloids at the cheek, chest and back area compared to the ears, upper limb and scalp regions.

Parameters	**Sum of squares**	**Df**	**Mean squares**	**F-value**	***P*-value**
Between groups	1024	1	1024	2.48199	0.137
Within groups	5776	14	412.57		
Total	6800	15			

Table 4B: Anova test for variance demonstrated F-value of 2.48199 with a *P*-Value of 0.137.

4.4: Surgical Excision Technique and Keloid Recurrence

Of the 90 patients, 45 with 56 keloids had surgical excision by intra-lesional excision technique (IL group) (Figure5, 6) and the remaining 45 with 58 keloids had excision by extra-lesional technique (EL group) (Figure7, 8). Pearson correlation test between both groups demonstrated anr-value of 0.9997. Anatomical locations of the keloids were the ears (42.8% IL, 39.6% EL), cheek (16% IL, 15.5% EL), abdomen (10.7% IL, 13.7% EL) and chest (12.5% IL 13.7% EL)

(Figure 9). The differences in the proportions of keloid locations between both groups were not statistically significant. The surface areas of the keloids excised in the two groups were 8.95 cm^2 (IL) and 9.35 cm^2 (EL), and the difference was not statistically significant (*P*-value 0.909). Keloid recurrence after 3, 6, 12 and 24 months of follow-up occurred for 8, 9 , 9 and 9 patients (IL group) and 8, 9 ,10 and 10 patients (EL group) respectively (Figure 10). There was no statistically significant difference in the recurrence rate between the two group (*P*-value >0.05).

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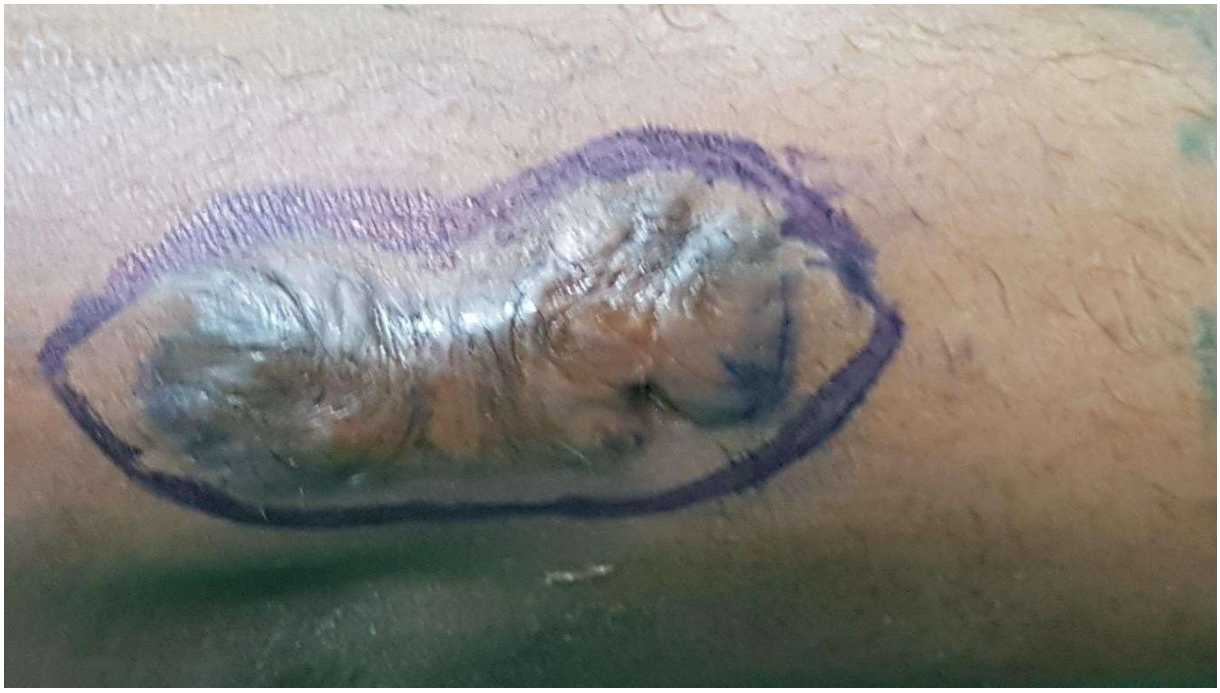


Figure 6: Marking for a keloid to be excised by extra-lesional (EL) technique. Note intended excision margin beyond the keloid into the normal skin.

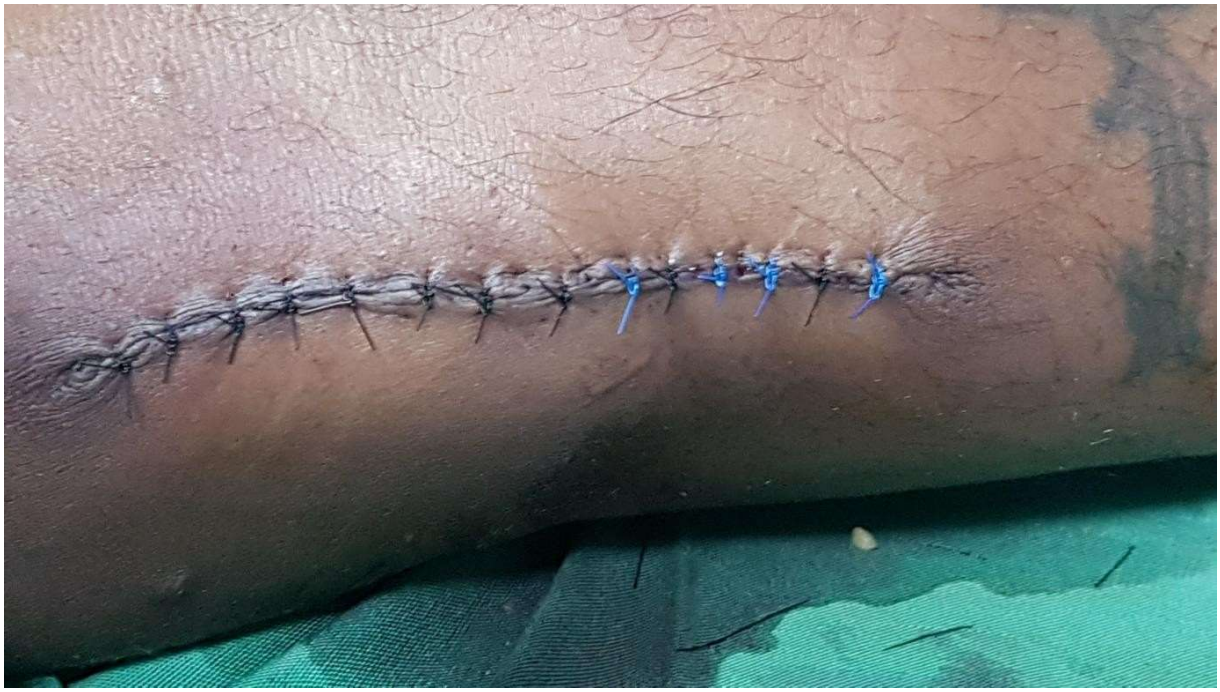


Figure 7: Extra-lesional excision of the keloids with primary wound closure. Note all keloid tissue has been excised.

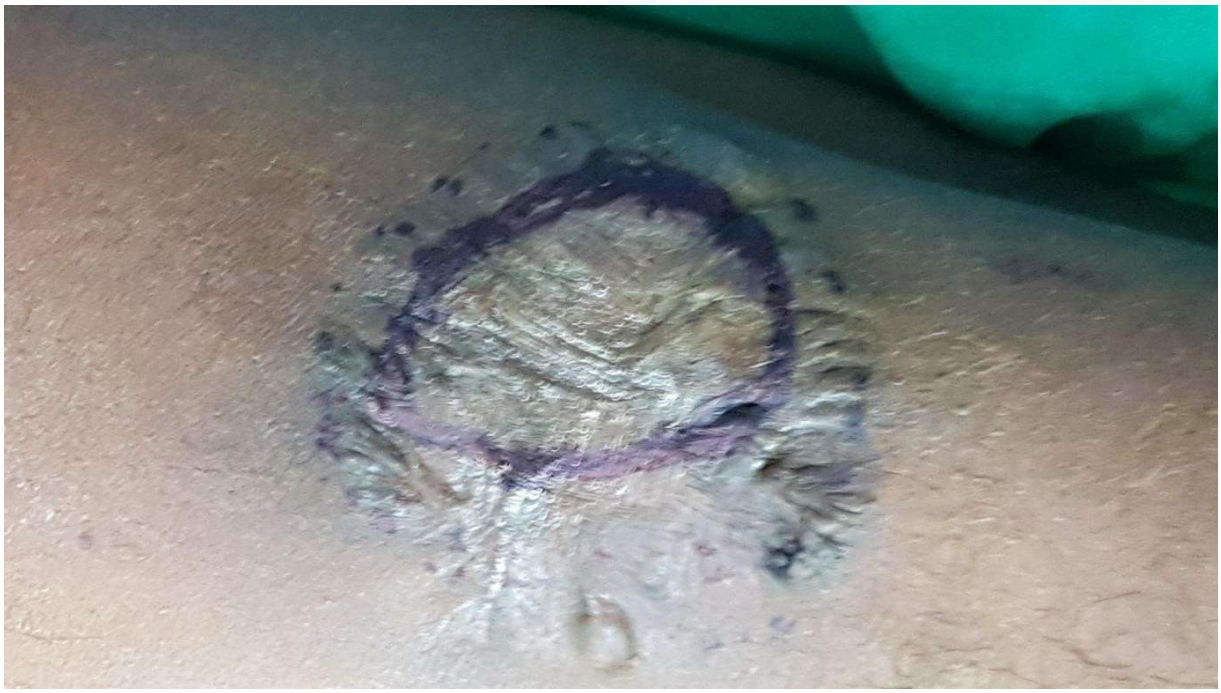


Figure 8: Marking for a keloid to be excised by intra-lesional technique. Note the intended incision line is within the keloid specimen.

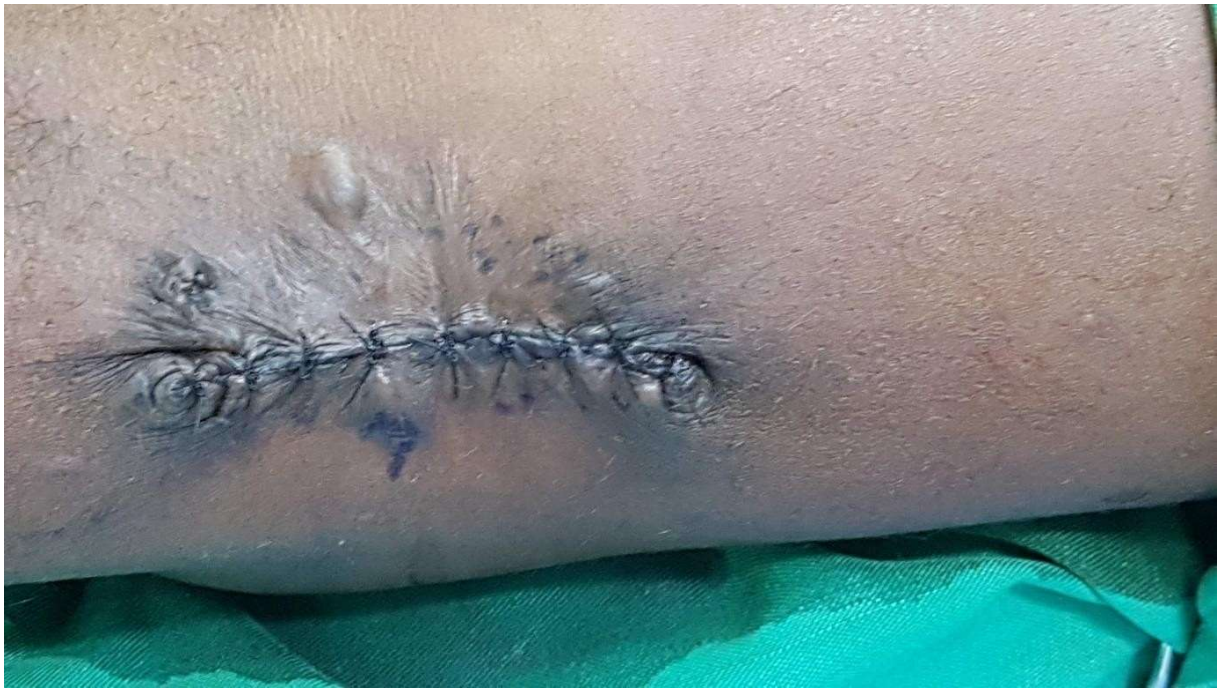


Figure 9: Intra-lesional excision of keloid tissue. Note the presence of residual keloid tissue at the end of surgery

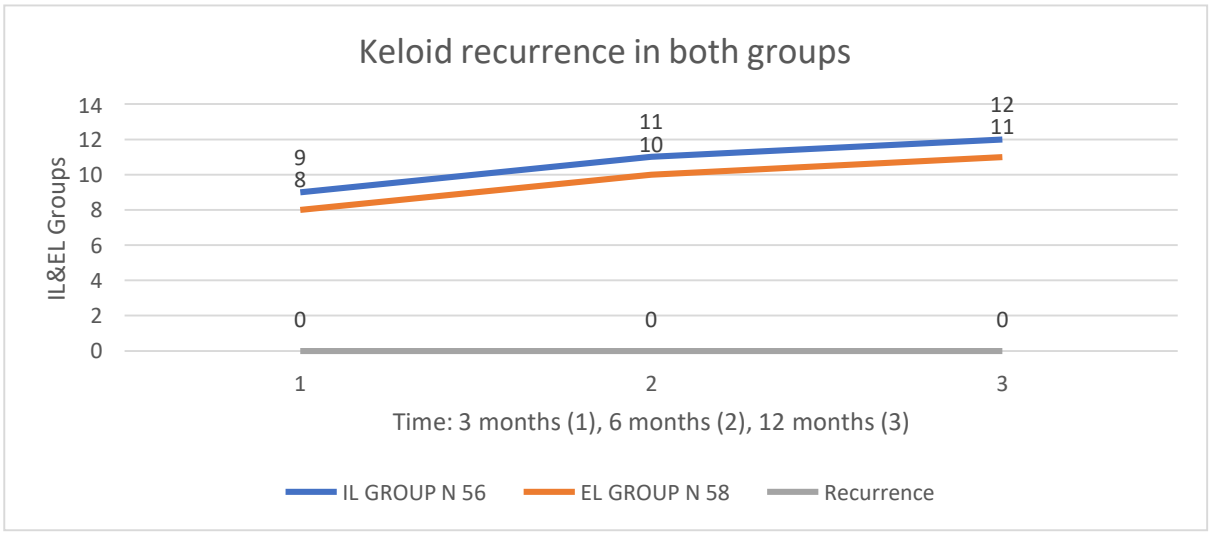


Figure 10: Summary of the recurrence rates.

The intra-lesional group had a higher recurrence rate 3, 6 and 12 months post-surgically than the extra-lesional group, although the difference was not statistically significant (*P*-value>0.05).

4.5: Blood Groups and Keloid Recurrence

The most common blood group for patients with Keloids and the control group with no keloids were blood group O at 51.3% and 49.2% followed by blood group A and B respectively. There was no statistical significance difference in the blood groups of patients who formed keloids and the control group (*p*-value >0.05).

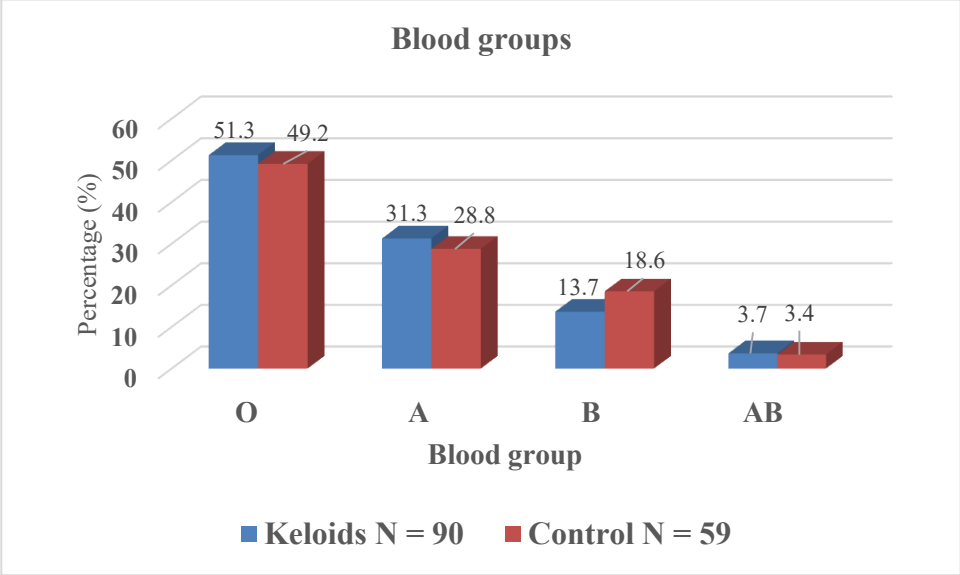


Figure 11: Blood groups in patients with keloids and the control group

Blood group	Total Count	Keloids N = 90	Control N = 59	P-Value (chi square Test)
		N (%)	N (%)	
O	75	46(51.3)	29(49.2)	0.552
A	45	28(31.3)	17(28.8)	0.480
B	23	12(13.7)	11(18.6)	0.175
AB	6	4(3.7)	2(3.4)	0.932

Table 5: Blood group of patients with keloids and the control group. There was no statistical significance difference between patients with keloids and the control group.

Comparison between blood groups in patients with KR and NKR patients demonstrated that keloid patients with blood group A were more prone to recurrence compared to the other blood groups (*P*-value =0.01) while patients with blood group B were least likely to have keloid recurrence (*p*-value =0.001).

Blood group	Total Count	Non-Récurrent(NKR) N = 71 N (%)	Recu rent (KR) N = 19 N (%)	P-Value (Chi square Test)
O	45	35 (50.0)	10 (55.6)	0.124
A	28	19 (29.00)	9 (38.9)	0.011
B	13	13 (17.7)	0	0.001
AB	4	4 (3.3)	0	0.516

Table 6 : Blood group and Keloid recurrence. Patients with blood group A were more prone to recurrence (*P*- value<0.05) while blood group B were least prone to recurrence (*P*-value <0.05).

4.6: Human Leucocyte Antigens (HLA) and Keloid Recurrence

A total of 139 patients had blood analyzed for HLA sub-types: 80 with keloids (computer generated randomly sampled) and 59 with no keloids. Of the 80 patients with keloids, 18 had keloid recurrence and 62 no recurrence as shown in the algorithm below:

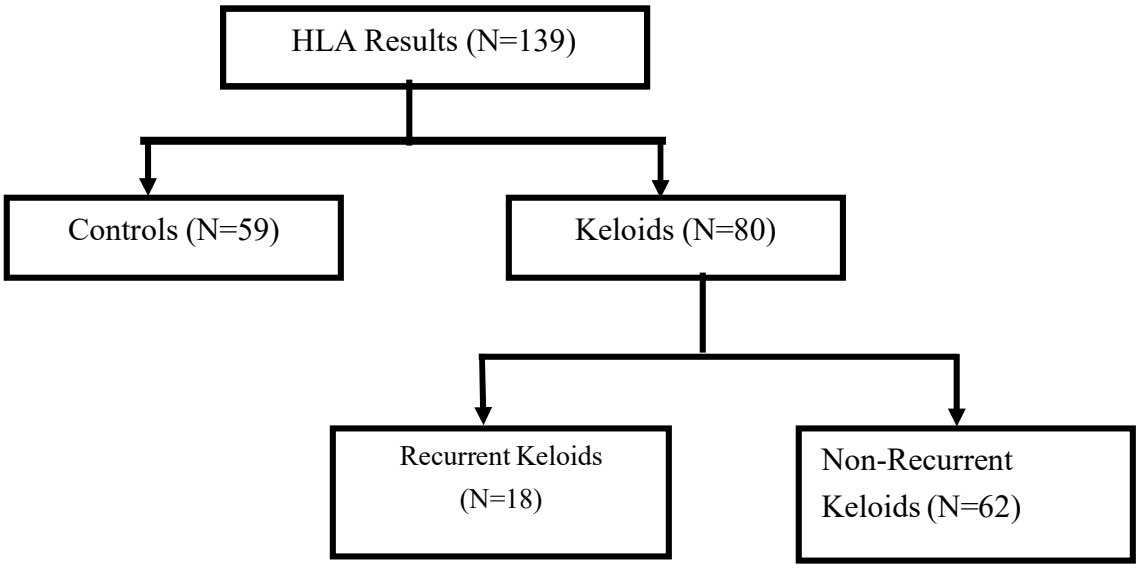


Figure 12: Flow chart summary for HLA sub-types in patients with keloids and control group

As seen in Table 7**,** there was a significance difference in several HLA alleles in patients with keloids and controls. Patients with keloids had a significantly higher positive alleles with DQA*01, DQB1*05, DQB1*06 and DRB1*15.

Allele	Keloids N = 80		Control N = 59		*P*-Value Chi square	Bonferroni correction *P*-value
	Allele +ve (%)	Allele −ve N (%)	Allele +ve N (%)	Allele −ve N (%)		
DQA1*01	83.8	16.2	44.1	55.9	<0.0001	0.0004
DQA1*02	5.0	95.0	13.6	91.5	0.129	
DQA1*03	8.8	91.3	13.6	86.4	0.415	
DQA1*04	7.5	92.5	5.1	94.9	0.733	
DQA1*05	51.3	48.7	47.5	52.5	0.732	
DQA1*06	6.3	93.7	6.8	93.2	1	
DQB1*01	3.8	96.2	1.7	98.3	0.637	
DQB1*02	10.0	90.0	8.5	91.5	1	
DQB1*03	22.5	77.5	23.7	76.3	1	
DQB1*04	20.0	80.0	20.3	79.7	1	
DQB1*05	55.0	45.0	30.5	69.5	0.006	
DQB1*06	55	45	0	100	<0.0001	
DQB1*07	1.3	98.7	0	100	1	
DRB1*01	20.0	80.0	27.1	72.9	0.415	

DRB1*02	2.5	97.5	5.1	94.9	0.650
DRB1*03	23.8	76.2	25.4	74.6	0.844
DRB1*04	11.3	88.7	6.8	93.2	0.557
DRB1*05	6.3	93.7	5.1	94.9	1
DRB1*06	3.8	96.2	0	100	0.086
DRB1*07	6.3	93.7	13.6	86.4	0.155
DRB1*08	7.5	92.5	6.8	93.2	1
DRB1*09	1.3	98.7	3.4	96.6	0.574
DRB1*10	2.5	97.5	0	100	0.508
DRB1*11	16.3	83.7	15.3	84.7	1
DRB1*12	3.8	96.3	3.4	96.4	1
DRB1*13	12.5	87.5	13.6	86.4	1
DRB1*14	6.3	93.7	6.8	93.2	1
DRB1*15	52.5	47.5	22.	78.0	0.004
DRB1*16	1.3	98.7	3.4	96.6	0.574

Table 7: HLA sub-types comparison between patients with keloids and normal controls. Keloid patients had significantly higher alleles of DQA*01, DQB1*05, DQB1*06 and DRB1*15 compared to the controls (*P* –values <0.05).After Bonferroni correction, HLA DQA*01 and DQB1*06 were still noted to be significantly higher among patients with keloids.

Allele	Non Recurrent Keloids (NKR) N = 62		Keloid recurrent (KR) N = 18		***P*-value Chi square**	**Bonferroni correction *P*-value**
	Allele +ve N (%)	**Allele −ve N (%)**	**Allele +ve N (%)**	**Allele −ve N (%)**		
DQA1*01	80.6	19.4	94.4	5.6	0.278	0.0006
DQA1*02	4.8	95.2	5.6	94.4	1	
DQA1*03	11.3	88.7	0	100	0.340	
DQA1*04	8.1	91.9	5.6	94.4	1	
DQA1*05	48.4	51.6	61.1	38.9	0.426	
DQA1*06	4.8	95.2	11.1	88.9	0.313	
DQB1*01	4.8	95.2	0	100	1	
DQB1*02	8.1	91.9	16.7	83.3	0.370	
DQB1*03	22.6	77.4	22.2	77.8	1	
DQB1*04	17.7	82.3	27.8	72.2	0.338	
DQB1*05	58.1	41.9	44.4	55.6	0.421	
DQB1*06	48.4	51.6	77.8	22.2	0.033	
DQB1*07	0	100	5.6	94.4	0.225	
DRB1*01	21.0	79.0	16.7	83.3	1	
DRB1*02	1.6	98.4	5.6	94.4	0.402	
DRB1*03	22.6	77.4	27.8	72.2	0.754	

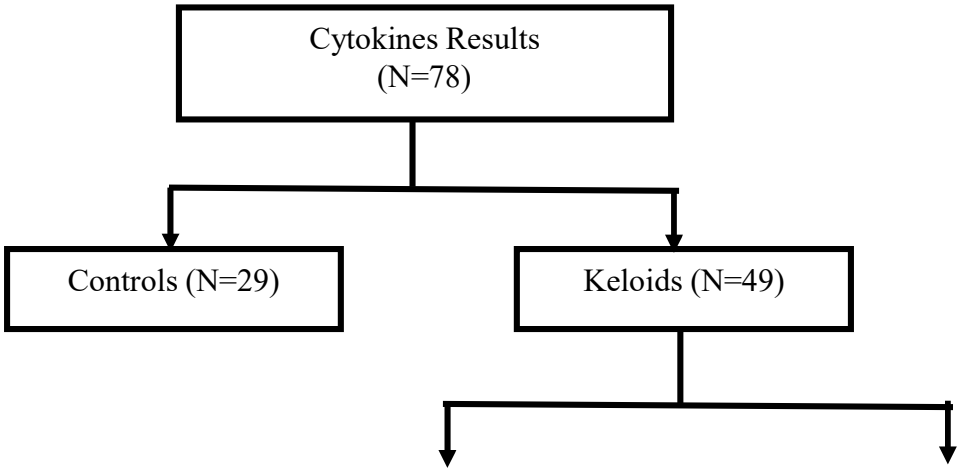
DRB1*04	9.7	90.3	16.7	83.3	0.413
DRB1*05	6.5	93.5	5.6	94.4	1
DRB1*06	4.8	95.2	0	100	1
DRB1*07	6.5	93.5	5.6	94.4	1
DRB1*08	6.5	93.5	11.1	88.9	0.612
DRB1*09	0	100	5.6	94.4	0.225
DRB1*10	1.6	98.4	5.6	94.4	0.402
DRB1*11	14.5	85.5	22.2	77.8	0.475
DRB1*12	4.8	95.2	0	100	1
DRB1*13	14.5	85.5	5.6	94.4	0.442
DRB1*14	6.5	93.5	5.6	94.4	1
DRB1*15	58.8	45.2	44.4	55.6	0.593
DRB1*16	1.6	98.4	0	100	1

Table 8: HLA sub-types analysis between patients with keloid recurrence (KR) and those without (NKR) revealed statistical significance difference with HLA allele DQB1*06.

The Bonferroni correction however revealed that the difference was not of statistical significance.

4.7: Cytokines and keloid recurrence

A total of 49 patients with keloids and 29 patients (both computer generated randomly sampled) with no keloids had their blood assayed for 28 cytokines using the Bioplex Elisa technique. Out of the 49 keloid patients studied, 11 patients had keloid recurrence while 38 patients had no recurrence. The algorithm below summarizes the findings:



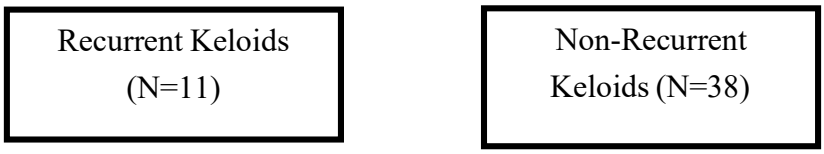


Figure 13: Flow chart summary for Patients assayed for the various cytokines

Twenty-eight cytokines were assayed during the study using theBioplex Elisa technique (See Appendix3). Of the assayed cytokines, there was a statistical significance difference with 15 cytokines between the two groups of patients. Of the fifteen cytokines, eleven were significantly elevated in patients with keloids with only four in patients without keloids (Tables 9 and10).

Among cytokines markedly elevated in the patients with keloids compared to the control was IL 4, 6 and13 all predominantly pro-inflammatory cytokines. Others elevated in keloid patients were IL-10, G-CSF, CCL4, HGF, IL-1RA and IL 2R.

	PWK			PNK		
	Min	**Max**	**Mean**	**Min**	**Max**	**Mean**
IL-1β	4	22	10.51	6	379	24.07
G-CSF	0	873	101.65	0	543	61.45
IL-6	7	1964	60.57	7	60	20.07
IL-12	0	311	95.57	19	367	102.45
CCL5	1000	9999	2831.00	1359	23370	4328.28
CCL11	20	155	63.65	3	352	72.83
IL-17A	0	35	6.59	0	37	4.45
CCL3	0	794	136.39	0	315	78.62
GM-CSF	1	631	21.39	3	27	7.38
CCL4	0	796	209.12	0	793	179.38
CCL2	67	1215	526.37	0	2300	571.59
IL-15	0	401	49.49	2	499	53.97

IL-5	0	33	6.08	0	16	5.62
HGF	0	3483	502.53	0	1882	333.62
VEGF	0	16	1.78	9	8	1.48
IFN-γ	0	10	0.90	0	12	1.31
IFN-α	0	195	27.04	0	229	24.21
IL-IRA	77	4783	1118.20	37	1939	705.69
TNF-α	0	24	5.37	0	16	4.14
IL-2	0	122	23.22	0	329	32.28
IL-7	0	126	15.94	0	79	15.28
IP-10	0	27	11.59	1	25	9.72
IL-2R	0	1412	377.31	0	1208	248.31
CXCL9	61	563	201.06	9	557	202.52
IL-4	11	317	56.80	11	94	43.17
IL-8	0	195	22.57	0	104	23.21
FGF-Basic	0	40	38.53	0	62	24.48
EGF	0	416	63.02	0	355	88.76

Table 9: Cytokines mean and range in PWK (keloid group) and PNK (control group). Note more than 11 cytokines were significantly elevated in PWK compared to only 4 in PNK group.

	Keloids (PWK)				**PNK**				***P* –value**
	Min	Max	SD	Mean	Min	Max	SD	Mean	(T-test)
FGF-Basic	0	40	14.52	38.53	0	62	18.69	24.48	0.026
G-CSF	0	873	202.87	101.65	0	543	133.06	61.45	<0.001
IL-10	0	3185	452.79	83.47	0	119	25.00	16.38	<0.001
IL-13	0	773	108.42	32.20	4	53	11.95	18.14	0.026
IL-6	7	1964	280.04	60.57	7	60	11.91	20.07	<0.001
CCL3	0	794	177.32	136.39	0	315	92.50	78.62	<0.001
GM-CSF	1	631	89.58	21.39	3	27	5.78	7.38	0.026
CCL4	0	796	161.52	209.12	0	793	160.63	179.38	<0.001

HGF	0	3483	585.41	502.53	0	1882	376.14	333.62	<0.001
IL-2R	0	1412	326.31	377.31	0	1208	239.33	248.31	<0.001
IL-4	11	317	51.37	56.80	11	94	17.35	43.17	0.031

Table 10A : Cytokines significantly elevated in patients with keloids (PWK) in comparison to control (PNK) group. Note inflammatory cytokines such as IL -6, IL-10, IL-4 and IL -13 were all significantly elevated in PWK group.

Anova test for Variance

Parameters	Sum of squares	Df	Mean squares	F	P-value
Between groups	15766.99	1	15766.99	0.857	0.0366
Within groups	367815.75	20	18390.79		
Total	383582.75	21			

Table 10 B: Comparison of cytokines in PWK AND PNK groups was determined using ANOVA showing that the difference was of statistical significance.

Comparison between cytokines that were significantly elevated in patients with keloid recurrence (KR) and those without (NKR)revealed CCL3, CCL4,CCL2, HGF, IL -IR A, IL-2R and IL -4 to be more elevated in patients with keloids recurrence than those without.

	Recurrent Keloids (KR) Mean	Non-Recurrent Keloids (NKR)Mean	*P*-value (T-test)
CCL3	181.18	123.42	<0.001
CCL4	248.82	197.63	<0.001
CCL2	696.55	677.11	<0.001
HGF	722.82	438.76	<0.001
IL-IRA	1205.45	1092.95	<0.001

IL-2R	452.45	355.55	<0.001
IL-4	81.82	49.55	<0.001

Table 11: Cytokines significantly elevated in patients with Keloids recurrent (KR) than none keloid recurrent (NKR)

4.8: Keloids Histology and Recurrence

Keloids specimens were analyzed for cellularity to determine whether they influenced recurrence. The analysis was both by light microscopy for all slides and immune-histochemical stains for a selected slide. Both techniques demonstrated keloids to have high densities of inflammatory cells such as macrophages, mast cells and fibro-blasts (Figures 14 and15).

Figure 14 A-C

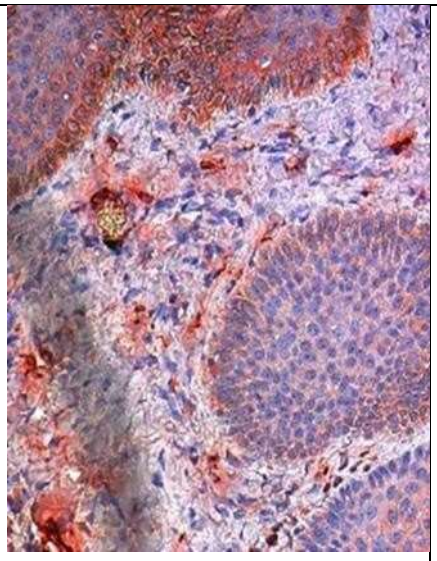


Figure 14A

Figure14B

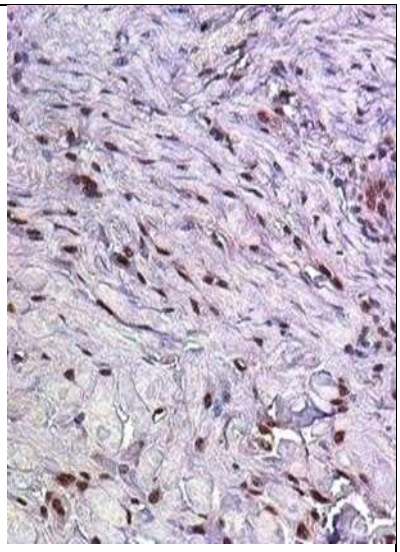


Figure 14C

Figure 14 A:Anti-Tyrptase positive mast cells in keloid specimen at x 100 magnification

Figure 14 B: Anti-Chymase positive mast cells in keloid specimen at x 100 magnification

Figure 14C: CD163 Positive macrophages in keloid specimen at x 100 magnification

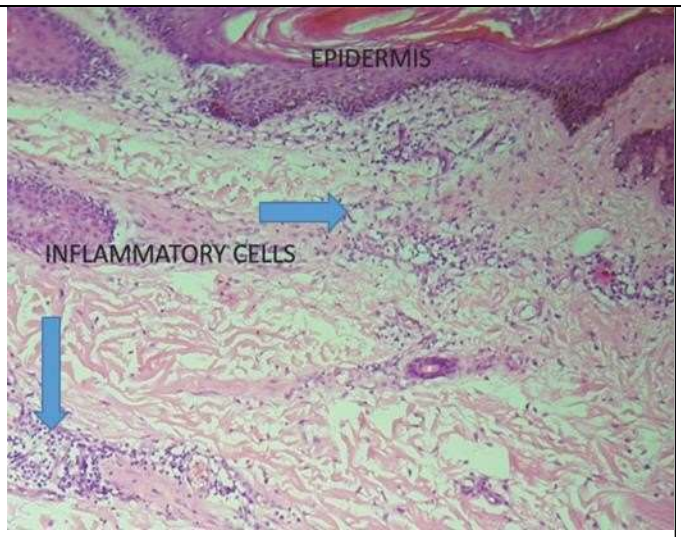


Figure 15A

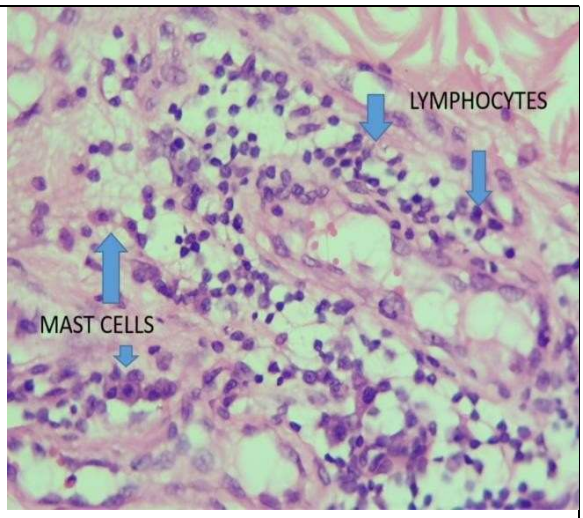


Figure 15B

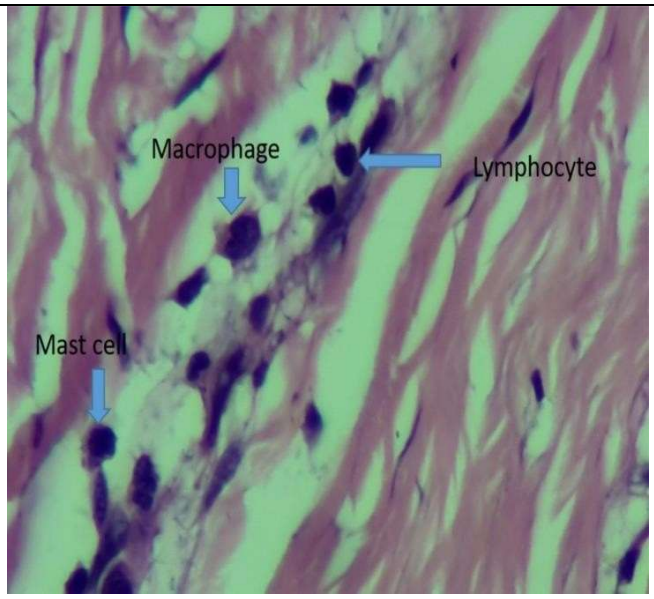


Figure 15C

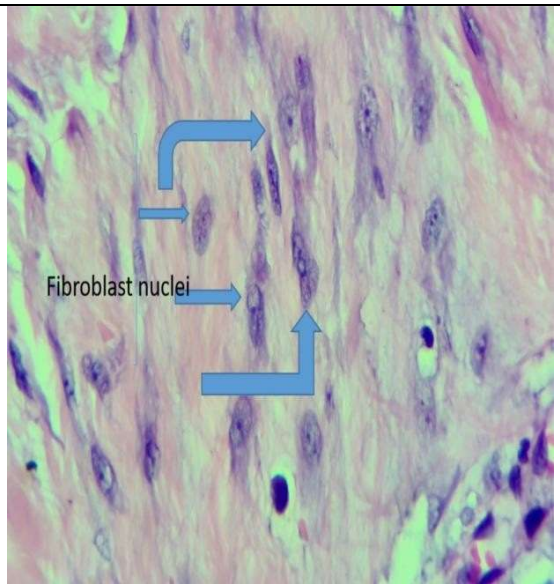


Figure 15D

Figure 15A.B.C.D

Figure 15 A: Haematoxylin and Eosin (H&E)stains of keloids At x100 magnification shows abudant inflammatory cells

Figure 15 B:H&E Stains x200 magnification demonstrate mast cells and lymphocytes

Figure15 C:H&E Stains at x200 magnifictaion demonstrates mast cells , lymphocytes and macrophages

Figure 15 D:H&E at x200 demonstrates fibroblasts

About 60 percent of the keloid slides studied had macrophages of less than 50 /HPF and 4 % had more than 100/HPF. Thirty-seven percent of the specimens had mast cells of more than 100/HPF, with only 17 % having less than 50/HPF. Fifty-two percent of specimens had lymphocytes count less than 50/HPF, with 27 % of specimens having fibro-blasts more than 100/HPF (Figure 16).

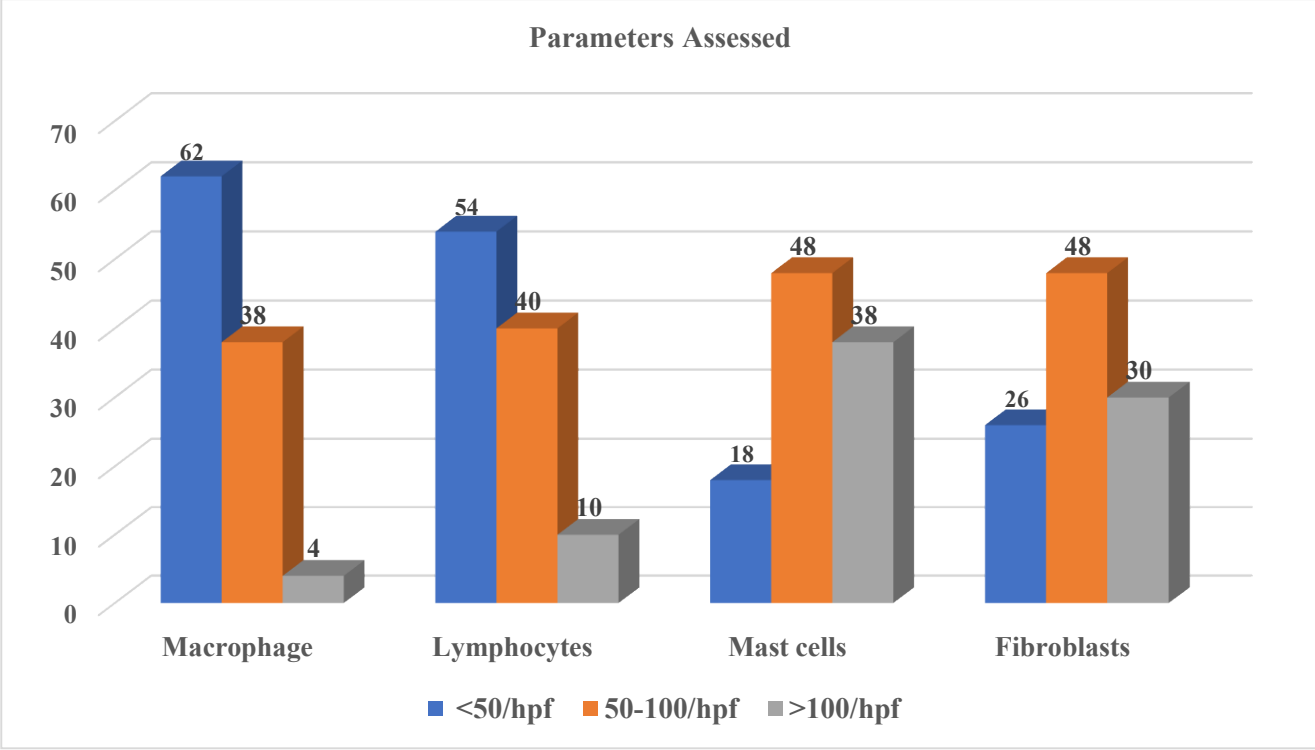


Figure 16: Summary of inflammatory cells and fibro-blasts counts in keloid specimens

Assessment of histological parameters in each keloid 75 percent of slides had a mast cell count of more than 50 per HPF.

Parameters	<50/hpf	50-100/hpf	>100/hpf	*P*-value Chi square test
Macrophage	62	38	4	0.000
Lymphocytes	54	40	10	0.000
Mast cells	18	48	38	0.001
Fibro-blasts	26	48	30	0.019
Parameters	<5/hpf	5-10/hpf	>10	
Capillary	30	56	18	0.000

Table 12: Summary of fibro-blasts and inflammatory cells in keloid specimens: Most of the specimens had elevated macrophages, lymphocytes, mast cells and fibro-blasts counts

Sixty-seven percent of keloids that recurred had lymphocytes count of more than 50 per HPF compared to 37 percent of keloids that did not recur (*p*-value 0.0064). Seventy-one percent of keloids that recurred had macrophages count more than 50 /HPF compared to 26 % of keloids that did not recur (*p*-value 0.05). Eighty-two percent of keloids that recurred had a mast cell count of more than 50 per HPF compared to 83 % of the keloids that not recur (*p*-value 0.128). Ninety-four percent of keloids that recurred had fibro-blast count more than 50/HPF compared to 66% of keloids that did not recur (*p*-value 0.005). The sensitivity and specificity of lymphocytes count of more than 50/HPF in the recurrence of keloids was 48 and 81% respectively. The macrophage counts of more than 50/HPF had a sensitivity of 57 % and a specificity of 83 % with mast cell sensitivity of less than 32 % and specificity of 33%. The fibro-blasts count of more

than 50/HPF has a sensitivity to determine the recurrence of keloids and a count of less than 50/HPF has a sensitivity of 41 percent with a specificity of 91%.

Parameter Assessed	Count/100 HPF	No recurrence N=84	Recurrence N=20	*P*-value Chi square test
Lymphocytes				0. 00064
	<50	58	2	
	50-100	18	16	
	>100	8	2	
Macrophages				0.000066
	<50	64	1	
	50-100	16	15	
	>100	4	4	
Mast cells				0.128742
	<50	10	2	
	50-100	44	17	
	>100	30	1	
Fibro-blasts				0.007191
	<50	58	2	
	50-100	16	10	
	>100	10	8	
Capillary density /hpf				0.1767
	<5	20	10	
	5-10	44	12	

Table 13A: Comparison of histological parameters assessment in patients who had keloid recurrence and those without recurrence revealed absolute lymphocytes, fibro-blasts and macrophages could influence the recurrence of the disease

Table 13B: Anova test for variance within the groups demonstrated a *P*-value of less than 0.05

Anova test for Variance

Parameters	**Sum of squares**	**Df**	**Mean squares**	**F-value**	***P*-value**
Between groups	1235.57	1	1235.57	9.537	0.005
Within groups	3368.29	26	129.55		
Total	4603.86	27			

Table 13B: Anova test for variance within the groups demonstrated a *P*-value of less than 0.005

4.9 : Keloid Mesenchymal Stem Cells and Recurrence

4.9.1: Mesenchymal Stem Cells between patients with keloids and the control group

A total of 61 patients with keloids (PWK) and a control of 32patients (PNK) had tissues analyzed for mesenchymal stem cells (MSC). The Pearson correlation test between both groups demonstrated a *P*-value of 0.9998. The mean MSC in PWK was 664.5 per gram of tissue with a range of 0 to 5180 and a median of 224. For the PNK, the mean was 665.8 per gram of tissue with a range of 0 to 4440 and a median of 91.70 (*P*-value 0.847) as presented in Table14.

Stem cell	PWK N = 61	PNK N =32	P-Value T test
Mean	664.5	665.77	0.987
Median	226	250	
SD deviation	900.45	1369.74	
Range	5190.00	4440.32	
Minimum	0	0	
Maximum	5190.00	4440.32	

Table 14: MSC density per gram of Tissue in PWK and PNK

There was no statistical significance difference in the mean density of keloids between the two groups. The percentages MSC in keloids and control were further analyzed by calculating a ratio of MSC to the total number of cells in the specimens. The mean percentage of MSC in keloids was 0.701% while in the control it was 0.182 % (*P*-value0.885). The median MSC for both groups of patients was 0.27 %. More than sixty-two percent of PWK had a MSC of more than 0.27 compared to 21.87 % of the control group (*p*-value<0.05) as seen in Table 15.

	Keloids N = 61	Control N = 32	P-value Chi square test
MSC % of the total cell count			
0 – 0.27	23 (37.70)	25 (78.13)	0.004
> 0.27	38 (62.30)	7 (21.87)	
Mean	0.701	0.182	0.885
Median	0.370	0.025	
SD Deviation	0.789	0.369	
Range	3.38	1.82	
Minimum	0	0	
Maximum	3.38	1.80	

Table15: Comparison of MSC percentages in PWK and PNK. Note the keloid group had a proportionately higher percentage than the control group with majority being greater than the median of 0.27 %.

The mean mesenchymal stem cells of KR were 848.1 compared to 578.3 for NKR (*P*-value<0.05). The median for NKR was 198.6 compared to 323 for KR.

Table 16 summarizes the above results

MSC	NKR N = 49	KR N = 12	P-value (T test)
Mean	578.52	848.13	<0.001
Median	198.66	323	
SD Deviation	1034.13	647.38	
Range	5190.00	2428.05	
Minimum	00	0.21	
Maximum	5190.00	2428.26	

Table 16: Comparison of the Mean and Median of MSC in Patients with KR and NKR; Patients with KR had a higher mean and Median compared to those with no recurrence.

The percentage of MSC in KR group was 1.123% compared to 0.427% in NKR (*p*-value 0.043). About 22 % of keloids with recurrence had MSC of less than 0.27 % compared to 78.13 % in keloids that did not recur. 78.2% of keloids that recurred were more than 0.27 % with only 21.9% of those that did not recur (*p*-value 0.022).

	NKR N = 49	KR N = 12	*P*-value Chi square test)
MSC proportions (%)			
0 – 0.27	21 (55.26)	5 (21.74)	
> 0.27	17 (44.74)	18 (78.26)	0.022
Mean	0.427	1.123	0.843
Median	0.310	0.910	

SD Deviation	0.435	1.032	
Range	2.12	3.38	
Minimum	0	0	
Maximum	2.12	3.38	

Table 17: MSC percentage of the total cell counts in NKR and KR. KR group had a higher percentage compared to the NKR group.

Regression Analysis

Multiple regression analysis was done to determine the strength of relations between family history , sex , anatomical location, blood group , keloid aetiology and recurrence .

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.687[a]	.643	.625	.72011

Table 18A: Model Summary for Multiple regression analysis

Analysis in table 18 above showed that the coefficient of determination (the percentage variation in the dependent variable being explained by the variation in the independent variables) R squared is 68.7% leaving only 31.3% unexplained variation.

- This means that 68.7% of the keloids recurrence was determined by the five variables that is Aetiology, Gender, Family history, Anatomic location and Blood group.
- 31.3% is unexplained variation due to other unknown factors including histology, HLA sub-types and cytokines.

Coefficients[a]							
Model		Unstandardized Coefficients		Standardized Coefficients	T	Odd Ratio	Sig.
		B	Std. Error	Beta			
1	(Constant)	2.335	0.466				
	Aetiology	0.279	0.085	0.698	3.2823	1.419	0.0115

	Gender	0.431	0.054	0.448	7.9814	0.651	0.005
	Family history	0.397	0.061	0.738	6.5081	0.576	0.0132
	Anatomic location	0.299	0.210	0.297	0.539	2.714	0.008
	Blood group	0.354	0.053	0.445	6.6792	0.793	0.016
a. Dependent Variable: Keloids recurrence							

Table 18B: Significance of the Coefficients of the Regression Model

The regression equation used was:

$Y= \beta_0+ \beta_1X_1+\beta_2X_2+ \beta_3X_3+ \beta_4X_4+ \beta_5X_5+$ €€

The established regression equation becomes:

$Y = 2.335+ 0.279X_1 + 0.431X_2 + 0.397X_3+ 0.299X_4+ 0.354X_5$

Interpretation

- Aetiology contributed to the recurrence of keloids (β=0.279; P=0.0021)
- Gender contributed to the recurrence of keloids (β=0.431; P=0.005)
- Family history contributed to recurrence of keloids (β=0.397; P=0.0132)
- Anatomic location contributed to recurrence of keloids (β=0.299; P=0.008)
- Blood group contributed to recurrence of keloids (β=0.354; P=0.016)

5.0 Keloid recurrence scoring system

Summarizing factors that influenced keloid recurrence in the study and in line with multiple regression analysis above, a keloid recurrence scoring system was developed as shown in the table below.

The highest score in the study 16 corresponded to a recurrence of 32 % and the lowest score of 6 corresponded to a recurrence rate of 11.2 percent as shown below.

Factors for keloid recurrent	**Percentage contribution to recurrence**	**Average Score**
Etiology		
Spontaneous	29	2
Infective	14	1

Traumatic	18	1
Gender		
Male	31	3
Female	10	1
Family history		
Familial	28.4	3
Sporadic	10	1
Anatomic location		
Cheek	33	2
Chest	33	2
Others	10	1
Blood group		
A	32	3
Other groups	10	1
Histology (inflammatory cells)		
High		3
Moderate		2
Low		1

Table19: Keloid recurrence scoring system.

The highest score in the study of 16 corresponded to a recurrence of 32 % and the lowest score of 6 corresponded to a recurrence rate of 11.2 percent.

CHAPTER FIVE: DISCUSSION

Keloid disease, one of the most difficult dermatological conditions to manage, has many modalities of treatment (Chike-Obi, 2009; Brissett, 2001). It is associated with high recurrence rates leading to dissatisfaction among patients. Factors that influence recurrence have been poorly understood making it hard for one to determine the outcome of patients' management. There are no guidelines or universally accepted protocols in the management of the disease due to paucity of data on the recurrence. This study was able to bring to the fore that keloid recurrence is influenced by multiple factors ranging from genetic, aetiological and anatomical

locations. These factors should therefore be taken into consideration while treating patients with keloids.

5.1: Patient Demographics and Gender on Keloid Disease and Recurrence

5.1.1: Age of Presentation

Keloids are more common in adolescents and young adults (Bayat, 2004; Brown, 2008). In our study, more than 63% of the patients were less than 35 years of age with more than 95% of the patients being less than 50 years of age. Female patients had a younger age of presentation with more than 70% below 30 years compared to male patients at 55%.The modal age group for the female patients was 20-25 years while for the males 25-30 years indicating that female patients tend to form keloids at a younger age compared to their male counterparts.The percentage contribution to the total number of keloids for the ages between 15 to 30years was significantly higher for female than male patients, thus reinforcing the above findings as well.

Our findings reflect previous epidemiological studies, which have shown keloid disease to predominantly affect young age groups (Ramakrishna, 2014; Brown, 2008, Bayat, 2004). In a study amongst Jamaican patients with keloids, Bayat *et al.*(2004), found a mean age of 29.5 years. Ramakrishna (2014) in a series of 1000 patients in India had more than 65% of the patients with a mean age of less than 30 years. About 24 % of the patients were between 30 and 50 years of age.He, however did not compare the age of presentation between male and female patients. The reasons why female patients presented earlier than their male counterparts are not known. One may however speculate that this could be due to early onset of puberty. Another reason could be the ear-piercing practice that is predominantly common in females in early teenage years. Our study however did not demonstrate any significance differences in keloid recurrence based on the age of presentation.

5.1.2: Gender of the Patients and keloid recurrence

The male to female ratio in our study was 1:2 suggesting that keloids may be common in females as opposed to male. Different studies have come up with conflicting evidence in as far as patient's gender is concerned.Shaheen *et al* (2016) on risk factors in keloids among Syrian population showed a similar incidence among male and female patients. Sun *et al* (2014) in a study among Taiwanese cohorts found female patients to be more affected by keloids than their male counterparts. Davis *et al* (2013) on analysis of keloid treatment in the USA between 1990 and 2009 revealed 62.9% of the patients who were managed to be female. Bayat *et al.* (2004) in another study on site specifics of keloid location among patients in Caribbean had a female preponderance of 65%, which is closer to our findings. Kiprono *et al.* (2015) in a population-based survey on keloids in Kenya and Tanzania demonstrated the disease to be slightly more common in females than male patients. The reasons as to why the disease is more common in females than males is not known; it is however postulated that the prevalence could be higher in females due to the ear-piercing practice that is more rampant in females, for aesthetic and lifestyle reasons, as well as better health seeking behavior of the females.

There were significant differences in the recurrence rates of keloids between male and female patients. The recurrence rate for the male patients was 31% compared to 12% for the female patients. Reasons as to why male patients had a higher recurrence rate compared to their female counterparts are yet to be known. One may however speculate that testosterone hormones may influence keloid disease and severity. A study by Shin, *et al*. (2016), similar to our study findings, reported a higher recurrence rate among male patients compared to the female patients after treatment with radiotherapy. They also stated that other factors that contributed to the

recurrence were patient's age, radiotherapy beyond 24 hours of surgery, large surface area of the keloid and extra-lesional excision as compared to intra-lesional (Shin *et al.,* 2016). In our study, neither the size of keloids nor age influenced the recurrence of the keloids.

5.2: Clinical presentation as a determinant to keloid recurrence

5.2.1: Familial/Sporadic presentation of keloids and recurrence

Slightly more than 50% of patients had familial keloids with the remaining 48% being sporadic. Significant differences included the fact that familial keloids had a male to female ratio of 1:1while sporadic keloids had male to female ratio of 1:3. The male to female ratio of 1:1 probably suggests an autosomal dominance mode of inheritance that has been proposed for keloids (Glass, 2017, Marneros, 2001 and Clark, 2009). The relatively higher familial keloids in this study have been found in a number of studies done on patients of African descent (Kouotou, 2019, Bella, 2011 and Chen, 2006). Kouotou *et al.* (2019) in Cameroon found familial keloids to contribute to about 43 percent of all patients with keloids closer to what we found. On the other hand, Shaheen *et al.* (2016) in a study among Caucasians in Syria demonstrated familial keloids to comprise only 20 percent of all keloids in Caucasians patients. Why patients of African descent seem to have a higher prevalence of keloids among family members compared to other races is not known.

While there was no statistical difference in the two groups pertaining to keloid surface area and age of presentation, we observed that pain and pruritus were significantly higher in familial keloids group compared to the sporadic keloids group. In familial keloids, 98% indicated pruritus compared to 61% for sporadic (*p*-value0.001). Furthermore, there was a significant difference in pain proportions between the two groups as we observed that 60% of familial keloids had severe

pains compared to 40% in spontaneous causes (*p*-value0.04). The familial keloids group also had a higher pruritus and pain severity score although the difference was not statistically significant. Keloid recurrence among our patients was significantly higher in the familial group compared to the sporadic group at 26.9 and 18.5% respectively (*p*-value 0.026). These findings demonstrate that familial keloids were a more severe disease compared to the sporadic keloids probably due to a stronger genetic predisposition. Other studies have also shown that keloids recalcitrant to therapy with higher recurrence rates were observed in patients with a positive family history of keloids (Bayat, 2003).

5.2.2 Keloids Aetiology and recurrence

Majority of keloids were secondary to trauma (n=66) followed by infections (n=21) and spontaneous (n=17). Keloid recurrence was noted to be significantly higher in spontaneous keloids compared to the other causes of keloids, probably suggesting that it is a more severe disease and hence with a higher likelihood of recurrence. Spontaneous keloids are thought to result from trivial trauma and probably a reflection of the tendency for keloid formation. Spontaneous keloids have been reported mainly in association with syndromes such as Rubinstein-Taybi syndrome, Dubowitz syndrome and Noonan syndrome indicating a genetic predisposition (Jfri, 2015). They have also been seen more in atopic patients (Jfri, 2018).

5.3: Surgical excision techniques and keloid recurrence

Our patients were subjected to two modalities of surgical excision with either intra-lesional (IL) or extra-lesional (EL) to determine whether they could influence keloid recurrence. The mean age, keloid surface area and clinical factors, such as pruritus and pain, were virtually similar in the two groups. All patients were operated on with the same surgical team using similar surgical

techniques. Post-operative management and follow up were similar in both groups as well. Although pruritus score during follow-up was higher in the IL group than in the EL group, the difference was not statistically significant. The pain score, though higher in the EL group, was also not significantly different from that of the IL group. The recurrence rate was slightly higher in the IL than in the EL group. However, the difference was not of statistical significance.

The above findings suggest that keloid recurrence between the two modalities of treatment after one year of follow-up with post-excision superficial radiotherapy were virtually the same. Our findings differed from previous studies that had concluded that incomplete excision of keloids were associated with higher recurrence rates than complete keloid excisions (Shin, 2014; Ioannis, 2019; Tan *et al*., 2010 and Chong, 2018). Tan *et al*. (2010) found that keloids in patients who underwent incomplete excision had higher recurrence rates than those with completely excised margins. Their study was based on archived keloid specimens with clinical data captured retrospectively from records. The role of postsurgical adjuvant therapies and whether they could have influenced the outcomes were not well described. Further differences encountered during the study were the fact that data on symptoms, such as pain and pruritus were not captured making it difficult for one to decide whether these could have been affected by the type of excision. Chong Y *et al.* (2018) on auricular keloids demonstrated that patients with clear keloid margins had lower recurrence rates than those with involved margins. Their study was based on histological examinations of keloid specimens to determine status of the margins and correlated with recurrence. It is however not clear whether patients were subjected to any auxiliary therapies such as radiotherapy. Further, as in the Tan *et al.* (2010) study, little was mentioned about other symptoms, such as pain and pruritus, which are key complaints by the majority of patients in our population, and should be considered as part of the recurrence spectrum.

On the other hand, some studies have demonstrated good outcomes with intra-lesional excision of keloids. Donkor (2007) in a series of patients who had been operated on with intra-lesional excision followed by post-excision steroid injection, reported no keloid recurrence after two years of follow-up. Another study by Engrav *et al.* (2008) on the management of hypertrophic scars/keloids demonstrated better outcomes with intra-lesional excisions than with extra-lesional excision.

5.4: Blood group and keloid recurrence

Previous studies on patients' blood group and keloids had shown inconsistent results. While Ramakrishna *et al* (2014) and Shaheen *et al* (2016) demonstrated an association between blood group A and keloid formation, Abbas *et al* (2012) did not show any statistical significant differences between various blood groups and keloid disease. This study, similar to Abbas *et al* (2012), revealed that there was no statistically significant difference between blood groups in patients with keloids and the control group. The most common blood group in both groups was blood group O followed by A, B and AB, respectively. However, analysis between blood groups and keloid recurrence revealed that patients with blood group A were more prone to recurrence while blood groups AB were less prone to recurrence. This was a significant finding that had not been reported in any other study. The reason why blood group A could be more prone to recurrence could be derived from other clinical studies that have shown individuals with this blood group to be more prone to severe outcomes from inflammatory conditions such as rheumatic fevers,systemic lupus erythramatosis and COVID - 19 pandemic (Rita *et al*., 2020, Glimyarova *et al*.,2020).

5.5: HLA sub-types and their correlation to keloid disease and recurrence

There was an association between alleles DQA*01, DQB1*05, DQB1*06 and DRB1*15 with keloid formation in this study. Even further DQB1*06allele was associated with keloid recurrence. These findings were in keeping with a number of studies that had shown several alleles to be associated with keloid disease (Wen-Sheng, 2011; Brown, 2009 and McCarty, 2015). Interestingly, DRB1*15 seems to be common in most of the studies. Wen-Sheng (2011) found DQB1*0501, B*07-DRB1*15, DQB1*0503-DRB1*15 to be associated with keloid formation among Chinese Ham patients' cohorts. Another study by Brown *et al* (2009) found an association between HLA DRB1*15 and keloid formation in a patient cohort of Caucasians. However, in a Caribbean study of predominantly patients of African descent, Brown *et al.* (2009) found no association between HLA DRB*15 alleles and keloid patients. Interestingly HLA DRB*15 has also been associated with a number of auto - inflammatory fibrotic conditions such as multiple sclerosis and idiopathic pulmonary fibrotic diseases (Weatherby, 2002, Xue, 2011).

Our findings and those mentioned above seem to suggest that keloid pathogenesis and recurrence are influenced by the patient's immune system akin to auto - inflammatory diseases. The exact mechanism on how this is achieved is still not clear and probably requires further research. It is however postulated that certain alleles such as DRB1*15 may be more efficient at antigen binding and presentation to the antigen presenting cells following trauma or any injury, provoking a strong inflammatory and proliferative phase of wound healing, resulting in massive collagen deposition that may lead to keloid formation (Gergely, 2018). Evidence of the strong inflammatory response in keloid patients includes the fact that keloid patients in a number of studies have been shown to have elevated inflammatory cytokines compared to the normal

population suggesting a pro-inflammatory state of the body (Mohammed, 2007; Waldner, 2012; and Hui Xue, 2000). This was also demonstrated in this study. Majority of keloid specimens have also been shown to have a high concentration of inflammatory cells such as macrophages, lymphocytes and mast cells (Hessel, 2019). Further, normal fibro-blasts *in vitro* subjected to inflammatory cytokines such as interleukin 6 have responded by massive synthesis of collagen fibres. These activities were stopped by antibodies against IL-6 implying that abnormal fibro-blast activity in keloid patients is secondary to inflammatory response by cytokines (Mohammed, 2007; Waldner, 2012; Hui Xue, 2000). Our finding of the association of HLA DQB1*06 with keloid recurrence requires further studies given that this particular allele has also been associated with other fibrotic conditions such as sarcoidosis (Haoxiang, 2017).

5.6: Cytokines in keloid disease and recurrence

Majority of studies done to demonstrate cytokines levels in patients with keloids have largely been *in vitro* or limited to a small sample size, and that makes it hard for one to conclude whether they do play a role in keloid pathophysiology (Mohammed, 2007; Waldner, 2012; Hui Xue, 2000). A significant finding from this study was the fact that more than half of inflammatory cytokines assessed were higher in patients with keloids than in the control group, strongly supporting the fact that inflammation plays a critical role in keloid formation and recurrence. Among them was IL-6 that has been widely noted to play a critical role in the pathogenesis of various diseases (Anne, 2020; Ciccarelli, 2013; Toshio, 2010; Galeotti, 2011 and John, 2001). It is considered to be one of the most potent inflammatory cytokines. In our study, it was significantly elevated in patients with keloids with a mean that was statistically significant. Quonzhou *et al.* (2009) also found IL- 6 to be greatly increased in keloids than in normal

skin.They further demonstrated the ability to reproduce keloid - like tissue in immune-compromised rats by using keloids derived stem cells and IL- 6. This activity was stopped by antibodies against IL-6 suggesting a possible role of these anti-bodies in the treatment of keloids. McCauley *et al.* (2010) in another study showed monocytes of patients who form keloids produced large amounts of IL- 6 compared to normal patients. Hui Xue *et al.* (2000) demonstrated an elevation in the expression of IL-6 genes in the keloid fibro-blasts as compared to the normal fibro-blasts. The significance of the above findings in the management of patients can be postulated from the fact that anti-bodies against IL -6 cytokine receptor, Tocilizumab, which inhibits IL-6 binding to IL-6R, have successfully been used in the treatment of Castle man's disease and rheumatoid arthritis both auto-inflammatory conditions with high IL-6 levels (Enes, 2015). Comparing IL 6 levels in patients with keloid recurrence and those with no recurrence did not show any significant difference, implying that the absolute levels of IL 6 may not influence the severity of keloid disease and the likelihood of recurrence. Another cytokine with intense pro-inflammatory activities that was significantly elevated in not only keloid patients but also those with recurrence were IL-4. IL -4 has been shown to be one of the key cytokines in allergic conditions such as asthma and atopic dermatitis (Jonathan, 2019; Beer, 2008). Interestingly, population-based studies have demonstrated keloid prevalence to be higher in patients with these conditions probably suggesting a similar pathophysiology (Xuechuan, 2017). IL -4 stimulates B-lymphocytes to secrete immunoglobulin E (IGE, henceforth) as well as up regulate IGE receptors on mast cells and basophils (Jonathan, 2019; Beer, 2008). It also promotes cellular inflammation through vascular cellular adhesion molecules (VCAM, henceforth), promoting migration of T-lymphocytes, basophils and eosinophils from the intra-vascular compartments. Further activities are by promoting differentiation of naïve T-helper cells

to T-helper 2 cells that secrete IL-4. IL-5, IL-9 and IL-13 all critical in inflammatory response in wounds (Jonathan, 2019; Beer, 2008). T-Helper cells subjected to IL-4 have less apoptotic activities, a fact that probably explains a higher T-Helper2 cells ratio in keloids compared to normal skin and hypertrophic scars (Beer, 2008). Interleukin 13, another key inflammatory cytokines that has synergistic effects with IL-4, was also significantly elevated in patients with keloids. Similar findings were noted by Diaz *et al* (2014) who found keloid lesions to have increased signaling of IL 4 and 13 compared to the normal skin. They further demonstrated resolution of keloid symptoms in patients who were given IL 4 receptor antagonists, Dupilumab that is also used to treat eczema and other allergic conditions.

Both Macrophages inflammatory factors alpha (CCL3, MiPa) and Macrophages inflammatory factor beta (CCL4, MiP b) were significantly elevated in patients with keloids and keloid recurrence. These factors released by macrophages and other inflammatory cells have a strong pro-inflammatory effect in the body. Their role in keloid pathophysiology and recurrence has not been conclusively established. They stimulate monocytes, macrophages and fibro-blasts to release pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α. Others factors that were elevated in keloid patients compared to the controls were granulocytes colon stimulating factors (G-CSF) and granulocytes macrophages, colon stimulating factors (GM-CSF). They both play a big role in the body's innate immune response. Their role in keloid disease progression and recurrence needs to be evaluated further given the potential they could play in the treatment of the disease (Xiangwen, 2020). There were no statistical significant differences between patients with keloids and the control in reference to the TNF-α.IL -8, IL-12 and IFN-γ. Though TNF-α. was high in patients with keloids than the control group, the difference was only marginal.

However, McCarty *et al.* (2010) in the assay of blood mono-cytes in keloids and normal patients demonstrated elevated TNF-α in patients with keloids. Further, anti-TNF-α.antibodies topically injected in keloids showed a reduction in keloid size and pruritus (McCarty *et al*., 2010),

5.7: Keloid histology as a determinant of recurrence

We analyzed inflammatory cells, fibro-blasts and vascularity of keloids as variables that could influence recurrence of keloids. Important findings in the study were that most keloids had elevated inflammatory cell population of macrophages, lymphocytes, and mast cells, an indication that inflammation plays a critical role in keloid pathogenesis. Also elevated were the fibro-blasts. Regarding the inflammatory cell count, mast cells were the most elevated with a count of more than 50 per HPF in at least 87 percent of the slides analyzed. Equally raised were fibro-blasts with at least 75 percent of slides with more than 50 per HPF. Macrophages were the least elevated, with more than 60 percent of slides with counts less than 50 per HPF. These findings were consistent with several studies that have demonstrated elevated inflammatory cells in many keloid specimens (Boyce, 2001; Martin, 2009 and Hessel, 2019). Ali *et al.* (2019), in a study in Kenya, demonstrated high inflammatory cell counts in most keloid specimens. Bagabir R.*et al.* (2012) showed not only an increase in T-lymphocytes but also a high CD4: CD8 ratio. In addition, aggregation of lymphoid tissues in keloid referred to as the keloid-associated lymphoid tissues, were observed in at least 15% of the histological specimens (Bagabir, 2001). Their study did not, however, correlate the histological findings with disease recurrence. Similar findings were noted by Boyce *et al.* (2001) who noticed that keloid specimens had a higher concentration of lymphocytes and macrophages than that of the normal skin. Beer *et al* (2000) found a high absolute count of mast cells not only in keloids but also in the normal scars and

hypertrophic scars. Analysis between keloids cell count and recurrence of the disease revealed a positive correlation between the absolute cell count of lymphocytes, macrophages and fibro-blasts with recurrence. Mast cells and vascularity of the keloids did not have any correlation with keloid recurrence. Of all variables assessed in the study, elevated macrophages of more than 50 per HPF had the highest sensitivity and specificity of 57 %and 83 % respectively in determining keloid recurrence. These could be a pointer to the influence of macrophages on the pathogenesis and severity of the keloid disease. A Lymphocyte count of more than 50 per HPF had a sensitivity of 48 % and a specificity of 81 percent, with mast cell count being the least sensitive and specific in determining keloid recurrence.Though morphological studies of the macrophages were not conducted in this study, previous studies had demonstrated macrophage Type 2 (M2) to be the predominant macrophage in keloids compared to Type 1 (M1) (Qi 2018). M2 sub-type is predominantly anti-inflammatory. They have also been shown to have a strong angiogenetic potential compared to M1, a factor that could be critical in keloid formation (Qi2018). They produce platelet-derived growth factor and fibro-blast growth factors that stimulate the proliferation of fibro-blasts and stimulate collagen synthesis that is critical in keloid formation (Xiangwen, 2020; Nadine, 2014; Qi, 2018; and Nang'ole, 2019). In addition, they are thought to stimulate lymphocytes activities in keloids, which could be responsible for chronic inflammation that is now thought to be an essential component of keloid disease (Sato, 2018, Nangole, 2019). The significance of keloid histology in the management and recurrence of keloids was previously thought to be of little importance (Liu, 2018; Lim, 2019). Park *et al.* (2015) on investigating the role of clinical-pathological factors of keloids and recurrence found out that there was no correlation between keloid histological features and recurrence of the disease. Their study, unlike this study, analyzed factors such as lymphocytic infiltration in the upper dermis, peri-vascular

lymphocytic infiltration in keloid, presence of epidermal cyst, presence of foamy histocytes and foreign body reaction. They did not consider the quantitative numbers of inflammatory cells or fibro-blasts as a confounding factor to keloid recurrence as done in this study. Gulamhuseinwala *et al*. (2008) on analysis of excised suspected keloid histology specimens concluded that with good clinical judgment, there was no need for histological analysis of the keloid specimen, whether for diagnosis or to assist with guiding treatment. This has been a notion in our centre and many other centers prior to this study with most keloid specimen not being subjected to histology. Our study findings however strongly advocate for routine analysis of keloid specimens with emphasis on the quantification of the inflammatory cells, especially macrophages and lymphocytes since they seem to influence recurrence.

A surprising finding in our study was the insignificance of absolute mast cells counts in keloid recurrence. Mast cell count was found to have the least sensitivity and specificity in determining the recurrence of keloids among the inflammatory cells. Mast cells had previously been thought to be critical in keloid formation. They were thought to be responsible for pain and pruritus in patients with keloid (Jumper, 2015; Ahuja, 2014). A number of studies had demonstrated not only high mast cell count in keloid patients but also in normal skin and hypertrophic scars, thus casting doubt as to whether the absolute mast cell numbers have any relevance in keloid scarring and recurrence (Boyce, 2001; Martin, 2009). In our study, more than 85 percent of patients had an absolute mast cell count of more than 50 per HPF. There was no statistical significance on the absolute number of counts and recurrence of the disease. Ammendola *et al*. (2013) found activated mast cells that were tryptase positive to be elevated in keloids than normal skin, probably implying that it was not the absolute number of mast cells that could be critical in keloid formation and recurrence, but the activated mast cells.

5.8: Mesenchymal stem cells as a determinant of keloid recurrence

The role of MSC in the pathophysiology and recurrence of keloids is not well defined. Though MSC and haematopoeitic stem cells have been implicated in keloids, it is not clear whether they do play a role in the pathophysiology of the disease or recurrence. MSC in tissues are thought to exist in two forms: MSC1 and MSC2. MSC1 is predominantly pro-inflammatory while MSC2 is anti-inflammatory (Watermann, R *et al*. 2010). Under the influence of local cytokines such as TNF-α.and IFN-γ, MSC differentiate into MSC2 that is predominantly anti-inflammatory while lack of these factors would result in MSC1 that is predominantly pro-inflammatory. The switch toward pro-inflammatory MSC1 phenotype or anti-inflammatory MSC2 phenotype may also depend on MSC stimulation through toll-like receptors (TLRs) expressed on their surface (Watermann 2010; Bommier *et al*., 2016). Stimulation of TLR3 leads to MSC2 of cells while TLR4 leads to the pro-inflammatory type, MSC1 (Bommier *et al*., 2016).

Our study demonstrated that keloids have a high percentage of MSC compared to the normal skin though we did not assay the phenotypes. Even further, keloids with recurrence had an even higher density of MSC and percentage of MSC than those without, suggesting that they could play a role in keloid disease processes. It is however difficult to state whether the high MSC in keloids could be primary to keloid formation or secondary to the disease process given that stem cells seem to be recruited from other parts of the body and be cited at sites of injury or inflammation. The cells home to damaged tissues and contribute to their 'repair' by secretion of cytokines, chemokines, and extra-cellular matrix proteins as well differentiating into proliferative cells. The anti-inflammatory cytokines produced by the mesenchymal stem cells include - interleukin 10, Nitric oxide and PGE2 (Bommie *etal.* 2016).

Manipulations of the local keloid 'cytokine environment' may thus influence the differentiation of stem cells into either MSC1 or MSC2 that may lead to desirable effects on keloid clearance, progression or recurrence. In line with this, is the fact that Imiquimod, a topical immune-modulator that functions through the toll-like receptor 7, known to up - regulate pro-inflammatory cytokines, including TNF-α, is known to reduce collagen production in fibro-blasts and has been tried as an adjunct to keloid treatment with mixed results (Berman, 2002). Further, several studies have demonstrated desirable effects on the use of either Adipose derived or haematopoeitic stem cells on treatment of keloids(Spiekman, 2014; Bommie, 2016; Fong, 2014 and Ya, 2017). Refinement of such studies may provide an alternative management strategy for keloids.

5.9: Keloids Recurrence Scoring System

Findings from this study demonstrate that keloids recurrence can be evaluated using a keloid recurrence scoring system. Multiple regression analysis done demonstrated that patient's gender followed by family history and blood group has a strong relation with keloid recurrence. These factors together with keloid etiology, anatomical location and histology were considered in the scoring system. Although other variables such as HLA sub-types, mesenchymal stem cells and cytokines did influence recurrence, we did not consider them in the system since they would be too expensive to be used routinely for keloid screening and management. Using the above scoring system, keloid recurrence rate could be estimated at between 11 and 32 percent. There is however need to validate the accuracy of this system in the future.

Further, patients with high likelihood of recurrence using this scoring system should be given a higher dose of post excision superficial radiotherapy to mitigate this from happening. Ogawa *et*

al. (2020) were able to reduce keloid recurrence rate to less than ten percent by giving higher doses of superficial radiotherapy to areas that were more prone to recurrence.

CHAPTER SIX: CONCLUSION

This study has demonstrated that keloid recurrence is influenced by many factors. Genetic factors that seem to influence recurrence are the patient's sex, HLA sub-types and blood group. Patients with blood group A, HLA sub-type HLABQ*06 and male sex are more prone to recurrence. Familial keloids as demonstrated in this study are also more prone to recurrence compared to sporadic keloids. Though clinical presentation and surgical management of keloids do not seem to influence recurrence, there is a correlation between keloid histological findings and recurrence. Keloids with high density of macrophages, lymphocytes and fibro-blasts are also more prone to recurrence. On the other hand, mast cell density and keloid vascularity do not seem to correlate with recurrence. Despite the fact that wide ranges of cytokines are elevated in patients with keloids, majority of them do not seem to influence keloid severity and thus the propensity to recurrence. There is however, a correlation between keloid mesenchymal stem cell density and percentage with recurrence of the disease. It is therefore critical that as many of these factors should be taken into consideration while managing patients with keloids to foretell the possibilities of recurrence. Keloid management should thus be 'customized' to the patient. Patients with higher likelihood of recurrence should be managed on a multimodality approach and monitored closely after surgery.

6.1: Recommendations

1. The findings of this study show that keloid recurrence is influenced by many factors. A keloid severity/recurrence scoring system as developed in this study would aid in the management of the condition and assist in predicting recurrence.

2. Keloid histology should be routinely done on keloid specimens since its findings have a prognostic value on the likelihood of recurrence. Parameters that should be looked for include the macrophages and lymphocytes densities.

3. Patients with keloids demonstrated significant elevation of many inflammatory cytokines strongly suggesting that inflammation plays a critical role in keloid pathogenesis. There is need to consider keloids as an auto-inflammatory skin disorder and not as a fibro proliferative disease. Potential pharmaceutical interventions targeting various inflammatory cytokines and receptors should be considered as a novel way of treating or preventing keloid formation.

4. Surgical management of keloids, whether intra-lesional or extra-lesional, does not seem to influence the recurrence of the disease. Either procedure can thus be used to treat patients with keloids. Factors to consider should be the size of keloids and the anatomical location since with extra-lesional excision, the scar seems to be better than intra-lesional excision while intra-lesional excision allows for ease of closure of larger keloids.

5. Though some studies seem to suggest that MSC may be beneficial in keloid treatment, this study demonstrated that MSC in keloids and those with recurrence were actually more than in the normal skin. There is need to carry out further studies to determine the role of mesenchymal stem cells in keloid formation and recurrence. Focus should be on the sub-types of MSC as well as their source.

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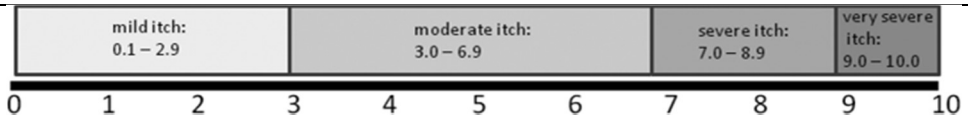
8.0 APPENDIX

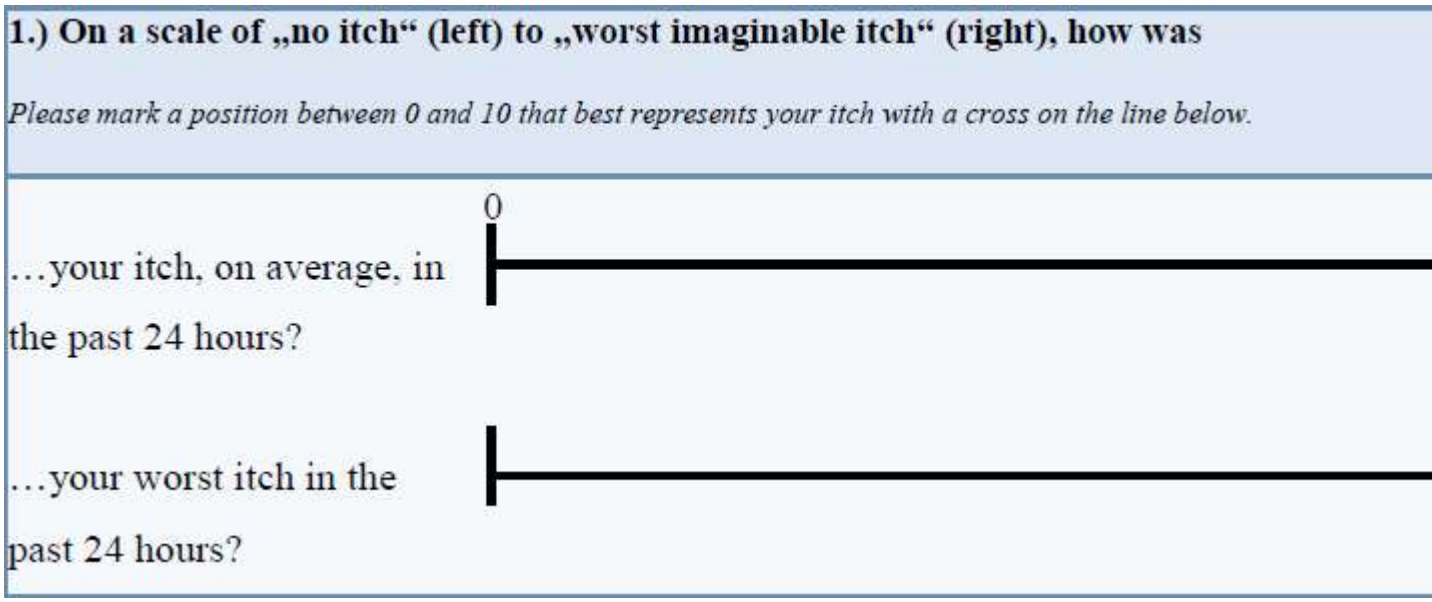
8.1APPENDIX 1: DATA COLLECTION FORM

PATIENT NUMBER				
Keloid Number				
AGE				
SEX				
WEIGHT				
BMI				
Family history of keloid				
Age of keloid onset				
Number of keloids at presentation				
Aetiology				
Anatomical location of the keloid				
Surface area of the keloid before excision				
Surface area after excision	1month	3 months	6monhts	12 months
Type of excision	1. Intralesional		2. Extralesional	
Pruritus				
				

DAY 0					
Day 7					
1month					
3 months					
6months					
12 months					
Pain score					
Day 0					
Day7					
1 Month					
3 months					
6 months					
12 months					
Histology					
Cellcount /hpf x100	0-50	50-100	100-150	>15-	
Macrophages					
Lymphocytes					
Mast cells					
Fibroblasts					
Collagen fibres /hpf	0-5	5-10	10-15	>15	
Vascularity of keloids /hpf	0-5	5-10	10-15	>15	
Macrophages					
Fibroblasts					

stem cells		
Lymphocytes		
Keloid status No reccurence,/reccurence		
Blood group type		
HLA sub-types		
Cytokines Assayed		
Stem cell Assayed	Quantity / gram of keloid tissue	

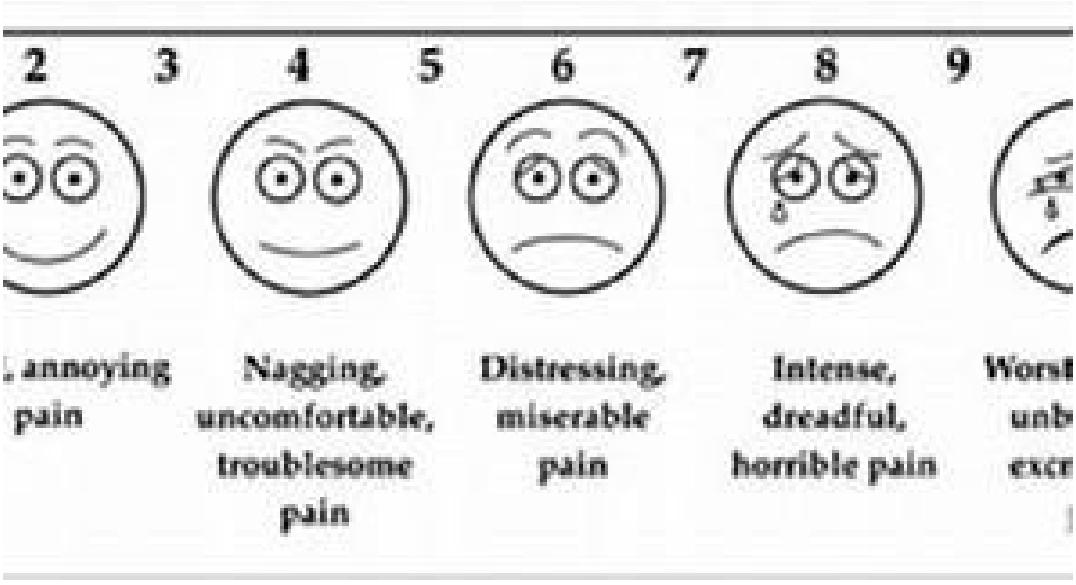
8.2 Appendix 2, Pruritus Visual Analogue Scale (VAS)



The VAS is a scale consisting of a 10cm long line and a single question. Along with the NRS, it is most commonly used in clinical trials for measuring itch intensity and features high reliability and concurrent validity. The left end point represents “no itch” and the right end point the “worst imaginable itch”.

It can be interpreted as follows: VAS 0= no pruritus
VAS < 3= mild pruritus
VAS ≥ 3-<7= moderate pruritus
VAS ≥ 7-<9 = severe pruritus
VAS ≥ 9= very severe pruritus

8.3: Appendix 3; Pain Analogue Scoring System



2

8.4 APPENDIX 4 .GENERAL PATIENT INFORMATION AND CONSENT FORM

8.4.1 English version

This is an informed consent form for persons aged 18 years and above as well as those below the age of 18 whose guardians/ next of kin/ parents allow to be included in the study whose title is Clinical pathological presentation of keloids and determinants of recurrence

Principal investigator: Dr Nangole Wanjala Ferdinand

Institution: School of Medicine, Department of surgery, University of Nairobi

Supervisors: Prof Omu Anzala, Prof Julius Ogengo

This informed consent has three parts

1. The Information sheet that seeks to give you details about the study
2. The certificate of consent to append your signature if you agree to take part
3. Statement by the principal researcher

A copy of the consent form shall be availed to you in full.

Part 1: Information sheet

My name is Dr. Nangole Wanjala, a lecturer at the School of medicine, University of Nairobi. I am conducting a research study titled 'heterogenisityof keloids and determinants of recurrence at Kenyatta national hospital'.

Keloidsis a common problem affecting many people especially those of dark skins. The problem with keloids is that they keep on recurring even after treatment has been offered resulting in the disappointment to the patients and the health providers. No studies have been done to determine why some keloids recur and others don't recur even in the same patient. There is thus need for studies to be done so as to evaluate on the possibities of why keloids do recur.This study aims to look at the histological composition of the keloids as a determinant to the recurrence of the keloids. Using the information derived from this study, conclusions will be drawn which may influence treatment practicesboth locally and internationally.

I would like to invite you to take part in this study. Participation is purely voluntary and you are allowed to consent either immediately after getting this information or after a period of consultation.

You are free to ask questions at any time regarding this study, or to seek any clarification from either myself or my research assistant. If you consent to participate in the study, some personal details as well as information concerning your condition will be sought.

You will then be randomly assigned a number between one (1) and fifty (200) recruited into one of the three arms of the study depending on thenumber you have been assigned to. Once assigned to either of the groups, you will be treated by surgical excision of the keloids. After the excision an injection of either triamcelonone or bleomycin will be infiltrated. The other option will be excision followed by post excision radiotherapy. The side effects of these medications will be explained to you.

There will be no difference in the quality of care between the various groups and neither are there any risks of being assigned to either arm.

You are guaranteed that all the information taken from you will be kept strictly confidential and will not be accessed by anyone other than the researchers and any other person authorized by the KNH/UON Ethics and research committee. This information will be coded with numbers such that only the researchers can identify you.

Participation in this study will be through a clinical interview and a clinical examination. Withdrawal from this study can be done at any stage and will not affect your treatment at this hospital.

This proposal has been reviewed and approved by the KNH/UON ERC which is a body that ensures the protection of persons like you that take part in research studies.

This approval has been granted after submission of the study proposal to the committee by the Chairman of the Department of Surgery, School of Medicine, University of Nairobi with the approval of a University supervisor.

In the event that you require any additional information or for any other purpose regarding this study, relevant contact details are listed below:

1. Dr Nangole Wanjala
   Department of Surgery
   School of Medicine
   University of Nairobi
   P.O. Box 19676-00202
   KNH, Nairobi

   Tel:0714342214

2. The Secretary
   KNH/UON Ethics and Research Committee (ERC)
   Tel no: +2542726300-19 Ext.44102
   P O BOX 20723-00202, Nairobi, Kenya

   Email: uonknh_erc@uonbi.ac.ke

3. Department of Surgery
   School of Medicine,
   University of Nairobi

   Tel:020-2726300

Part 2: Consent certificate

I .....freely give consent of myself /my proxy .....to take part in the research study carried out by Dr Nangole Wanjala Ferdinand, the nature of which he has explained to me. I understand that my participation in the study is purely voluntary and that I am free to withdraw this consent at any time. I also understand that withdrawing my consent will not affect the quality of care given to myself/my proxy at the Kenyatta National Hospital.

Signature of participant/Guardian/Next of kin……

Date……

I certify that the above consent has been freely given in my presence

Witness Name…….

Witness Signature……..

Date…….

Left thumbprint if participant illiterate (witness to countersign)

Part 3: Statement by the researcher

I confirm that the information relating to this study as contained in the information sheet has been accurately read to the participant. I confirm that I have ensured the understanding of its contents by the participant who understands that:

1. Declining to give consent or otherwise participate in this study will not affect the quality of care given at this institution
2. All information provided by the participant will be kept strictly confidential
3. The conclusions from this study may be used to influence local clinical practice

I further confirm that the participant has been allowed to seek clarification of all aspects of this study and that he/she has freely and willingly given consent. The participant has also been provided with a copy of the Informed consent form.

Name of researcher ……

Signature…….

Date……

8.4.2 Kiswahili version

Sehemu ya kwanza: Maelezo

Jina langu ni Dr. Nangole Wanjala ferdinand, mwalimu katika Kitivo cha masomo ya Udaktari, Chuo kikuu cha Nairobi. Ninafanya utafiti kuhusu' aina ya ufimbe ambao unaitwa keloid kwa kingereza. Ufimbe wa keloid ni ungonjwa ambao unawadhuru sana wale watu ambao wana ngozi ya kiafrika.Ni ungojwa ambao matibabu yake huwa ngumu sana na .huleta changa moto nyingi sana kwa wangonjwa na madaktari kwa jumla.

Kwa hivyo kuna haj ya kufanya utafiti ya kusaidia kujua kwa nini hu ufimbe una rudi kwa wangonjwa wengine na wengine haurudi baada ya matibabu.Utafiti huu amabao tunafanya unalenga kuangalia sehemu ya ile nyama ya keloid kunjukwaza na kuangalia kama kuna tofauti zake ambazo huenda zina changia kurudi kwa ufimbe baada ya kuttolewa na kutibiwa..wangojwa amabawo watanjiunga na huu utafiti watanagawanywa katika vikundi vitatu. Gikundi kimoja wata fanyiwa upasuaji na kupewa dawa aina ya triamcelonone. Gikundi kingine watu fanyiwa upasuaji na kupewa dawa aina ya bleomyicne.Gikundi cha mwisho watafanyiwa upasuaji ne kupewa radiotherapy. Wangonjwa wata chakuliwa katika hizi vikundi kwa njia ya uwazi na bila mapendelewo yoyote. Matibabu pia yatakuwa sawa na hukuna yoyote ambaye atabakuliwa.

Ningependa kukualika kujumuishwa kwenye utafiti huu. Kujumuishwa kwako ni kwa hiari na unayo haki kujiondoa kwenye utafiti huu wakati wowote. Idhini yako ya kujumuika unaweza kuipa maramoja baada ya kusoma nakala hii ama baada ya muda wa kufikiria. Unao uhuru wa kuuliza maswali yoyote kuhusu utafiti huu kutoka kwangu.

Baada ya kukubali kujumuishwa katika utafiti huu, utapewa nambari kati ya moja (1) na mia mbili), kisha kulingana na nambari uliopata utawekwa katika moja ya vikundi vitatu.

Hakuna tofauti yoyote katika hali ya matibabu katika vitengo vyote vitatu katika utafiti huu. Unaweza kujitoa katika utafiti huu wakati wowote bila kuadhiri matibabu yako.

Ukikubali kujumuishwa kwenye utafiti, maelezo yako binafsi pamoja na maelezo ya ugonjwa wako yatachukuliwa.

Utapatiwa hakikisho ya kwamba maelezo yote utakayotoa yatawekwa siri wala hakuna atakayeoona maelezo haya isipokuwa watafiti na watu waliokubaliwa na kamati ya uadilifu ya Hospitai kuu ya Kenyatta ikishirikiana na Chuo kikuu cha Nairobi. Nambari zitatumiwa badala ya majina ili kukinga maelezo yako.

Maelezo yatachukuliwa kwa njia ya maswali. Kujiondoa kwako hakutaadhiri kiwango cha matibabu utakachopatiwa katika hospitali hii.

Ruhusa ya kufanya utafiti huu umepatiwa kutoka Kamati ya Uadilifu wa Utafiti ya Hospitali kuu ya Kenyatta ikishirikiana na Chuo Kikuu cha Nairobi, kupitia Mwenyekiti wa Idara ya Upasuaji, Kitivo cha Masomo ya Udaktari, Chuo Kikuu cha Nairobi.

Ikiwa unahitaji maelezo zaidi kuhusu utafiti huu, tafadhali wasiliana na wafuatao:

1. Dr Ferdinand Wanjala Nangole
   Department of Surgery
   School of Medicine

University of Nairobi
SLP: 19676-00202
KNH, Nairobi

Simu: 0714342214

2. Katibu
   KNH/UON Ethics and Research Committee (ERC)
   Simu: +2542726300-19 Ext.44102
   SLP:20723-00202, Nairobi, Kenya

   Barua pepe: uonknh_erc@uonbi.ac.ke

3. Dr. Nang'ole Wanjala / Dr. Mark Awori
   Department of Surgery
   School of Medicine,
   University of Nairobi

   Simu: 020-2726300

Sehemu ya pili: Idhini

Mimi…….nimekubali kwa hiari yangu/hiari ya mgonjwa niliyemsimamia……..kujumuishwa kwenye utafiti unaoendeshwa na Dr Nangole wanjala, baada ya kupewa maelezo kamili na yeye. Ninaelewa kuwa kujumuika kwangu ni kwa hiari na nina uhuru wa kujiondoa wakati wowote. Naelewa kwamba kujiondoa kwangu hakutaathiri kwa vyovyote kiwango cha huduma nitakayopokea katika Hospitali Kuu ya Kenyatta.

Jina la mgonjwa/Msimamizi wa mgonjwa……

Sahihi……..

Tarehe……

Nimeshuhudia ya kwamba idhini ya mhusika imetolewa kwa hiari yake mwenyewe

Jina la shahidi.....

Alama ya kidole gumba cha kushoto(mgonjwa asiyejua kuandika – sharti shahidi kutia sahihi kando)

Sahihi ya shahidi……

Tarehe……

Sehemu ya tatu: Idhibati ya Mtafiti mkuu

Ninatoa idhibati ya kwamba maelezo kuhusu utafiti huu yametolewa kikamilifu kwa mhusika, na kwamba nimemsaidia kuelewa kwamba:

1. Kutotoa idhini ama kujiondoa kwenye utafiti huu hautaathiri kwa vyovyote kiwango cha matibabu atakayopata katika hospitali hii.
2. Maelezo yote yatakayotolewa yatawekwa siri.
3. Matokeo ya utafiti huu yanaweza kutumiwa katika kuchangia ujuzi wa kubaini ugonjwa unaochunguzwa.

Ninatoa idhibati pia ya kuwa mhusika amekubaliwa kuuliza maswali yoyote kuhusu utafiti huu na kwamba ametoa idhini kwa hiari bila kulazimishwa. Mhusika pia amepewa nakala ya stakabadhi ya idhini.

Jina la mtafiti ……

Sahihi……

Tarehe……

8.5: APPENDIX 5: HLA SUBTYPES ASSAY TECHNIQUE; DNA-Based Typing Method: SSP – PCR, SOPS

1. Patients' whole-blood specimens will be collected in anticoagulant bottles.
2. Sequence-specific PCR will be performed with allele-specific primers.
3. DNA Isolation will be done using an extraction kit.
4. Preparation of PCR Master Mix will be done by adding
- PCR Water, DNTPs, $MgCl_2$ and Taq-Polymerase
- Then DNA (75ng +/- 25ng per well)
- Aliquot PCR Master MixInto the two 96-well plates
5. PCR Amplification of the two 96-well plates then be done by preparing 10% gel and adding 0.5% ethidium bromide

6. Gel-electrophoresis will then be done by running at 220V for 15 minutes
7. Visualization of PCR products will be under UV illuminator and pictures taken for analysis of the results
8. Interpretation will be done using SSP Typing HLA allele table or software

NB: Qia-excel equipment is a one stop shop which can do both gel electrophoresis, visualize PCR products under UV illuminator and take pictures for analysis

PCR-SSP is used to determine class I HLA-A, B, C, and class II DR, DP, DQ locus types.

8.6 APPENDIX 6:Keloid Biopsy Processing and Staining, for mesenchymal stem cells, SOPS

This protocol is intended to allow processing and staining of Keloid biopsy cells in order for an assessment of the proportion of mesenchymal stem cells in the keloid biopsies.

Processing Keloid Biopsies

1. Prepare the collagenase solutions using the aliquot of 10mg/ml collagenase 2 solution in two 15 ml falcon tubes (1 aliquot is sufficient for one participant/visit)
    - To prepare a 1.0 mg/ml collagenase type 2 solution in the pre-labeled 15ml falcon tube: add 600 µl of 10 mg/ml collagenase to 5.4 ml of RO (No FBS present)
    - To prepare a 0.5 mg/ml collagenase 2 solution in the pre-labeled 15ml falcon tube: take 2 ml of RO and add 2 ml of 1.0 mg/ml collagenase 2 solution, mix by pipetting
    - You will have 4 ml of 0.5 mg/ml collagenase 2 and 4 ml of 1.0mg/ml collagenase 2 solutions ready to use.
2. The Keloid biopsies will be given to the lab team within 2 hours of collection time in one 50ml falcon tube containing 10ml RO and placed in wet ice.
3. Aspirate the medium (the 50 ml collection tube) carefully with 10 ml serological pipette (trying to get as much liquid out as possible), and discard. BE VERY CAREFUL NOT TO THROW AWAY THE KELOID BIOPSIES.
4. Transfer the keloid biopsies to one microcentrifuge tube labeled W-KL using a P200 pipettor (it creates some pressure that helps to hold the tissue in the point of the tip). Weigh the tube with keloid biopsy and record in a book.
5. Transfer the Keloid biopsies to three to four micro-centrifuge tubes labeled KL containing 750 µl 0.5 mg/ml collagenase 2
6. Incubate the biopsies on the vibrating heating block for 30 min at 37^{o}C at 1200 rpm to disrupt tissue.

7. In the intervening time, weigh the tubes without Keloid biopsies and the weight of keloid (W-KL-W-KL-1)
8. After 30 min move the tubes to the BSC and let the debris settle. Transfer the supernatant from 3-4 tubes of KL (which contains desired cells) into a 15ml tube (KL. Immediately add HI-FBS (10% v/v) to the supernatant to inactivate enzyme.
9. Manually disrupt/mash /jam the left over tissue in the micro-centrifuge tubes by using the P1000 tips.
10. Add 800 µl of 1.0 mg/ml collagenase solution to each tube, pipette up and down for 5-10 times, make sure the tissues can go through the P1000 tips again.
11. Incubate the tissue on the vibrating heating block for 10-15 min at 37ºC at 1200 rpm.
12. After 10-15 min, move the tubes to the BSC and PIPETTE WELL and transfer the liquid/debris to the previous amount of supernatant (stored on ice in the 15 ml tube).Add HI-FBS (10% v/v of the new supernatant volume added).
13. Place a sterile 70 µm cell strainer on top of pre-labeled 50 ml tube.
14. Transfer the cell suspension to the cell strainer using a 10 ml pipette. By pipetting up and down, make all liquid pass through the cell strainer into the 50 ml tube.
15. Complete the volume of the cell suspension filtered to 20 ml by adding this extra volume of R10 through the strainer to maximize cell recovery. Discard cell strainer.
16. Spin down the cells at 1600 rpm for 10 min. discard the supernatant. Resuspend the pellet with 10 ml of R10. Take out 10 µl into 90 µl of trypan blue (96 well plate)for cell counting.
17. Spin down 1600 rpm for 10 min, count cells using glass hemocytometer.
    - To calculate the cell yield =X*dilution factor *10^4*volume
18. Resuspend the KL pellet in X/10 ml volume of R10 (in this way, the cell concentration will be 1*10^6 per 100 µl). Keep the cells on wet ice until the surface staining starts (within 1 hour).

Live Dead Staining

1. After biopsy digestion and cellular isolation and counting, transfer the cells into 5ml FACS tubes, label tubes using alcohol-proof marker.
2. Add 2ml of PBS into each tube, wash cells by centrifuging at 1600 rpm, 22ºC for 5 minutes.
3. Discard supernatant by quickly inverting the tubes.
4. Thaw Live Dead Aqua antibody, add 1µl of L/D dye to 1 ml of PBS to make a stock L/D solution of 1:1000

5. Add 100 µlof the L/D solution for every 2 million cells, resuspend cells by pipetting gently up and down 3 times
6. Cap tubes and wrap with foil, incubate at 4ºC for 30 minutes, avoid light.

Surface Staining

1. During the intervening time, prepare surface staining master mix.
   (MSCs are characterized by the expression of stromal cell markers (CD73, CD105, CD44, CD29 and CD90) in the absence of hematopoietic markers (CD34, CD45 and CD14) and endothelial markers (CD34, CD31 and vWF).
   Positive Cocktail: CD105PerCP-Cy5.5, CD73APC, CD90FITC
   Negative Cocktail: CD45PE, CD34
2. After 30 minutes wash cells with FACS BUFFER TWICE and stain the cells with the surface markers. Incubate for 40 minutes at 4ºC.
3. Acquire cells on LSR2 within 24 hours.

8.7:Appendix 7; SOPS for Cytokines Assay

1. Add 200 µL of Wash Buffer into each well of the plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C). Mouse Metabolic Phenotyping Centers MMPC Protocols 09/23/13 3 of 5 page(s)
2. Decant Wash Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
3. Add 25 µL of each Standard or Control solution into the appropriate wells. Assay Buffer should be used for 0 pg/mL standard (Background).
4. Add 25 µL of Assay Buffer to the sample wells.
5. Add 25 µL of appropriate matrix solution to the background, standards, and control wells.
6. When assaying serum or plasma samples, use the Serum Matrix provided with the kit. When assaying tissue culture or other supernatant, use proper control culture medium as the matrix solution.
7. Add 25 µL of Sample (diluted one part serum or plasma to one part Assay Buffer) into the appropriate wells.
8. Vortex Mixing Bottle and add 25 µL of the mixed or premixed beads to each well. During addition of Beads, shake bead bottle intermittently to avoid settling.
9. Seal the plate with a plate sealer. Wrap the plate with foil and incubate with agitation on a plate shaker overnight (18~20 hours) at 4°C.

10. Allow reagents and assay plate to come to room temperature. Gently remove well contents and wash plate 2x following the instructions listed in the Plate Washing section.
11. Add 25 µL of Detection Antibodies into each well. Allow the Detection Antibodies to warm to room temperature prior to addition.
12. Seal, cover with foil, and incubate with agitation on a plate shaker for 1 hour at room temperature (20~25°C). Do not aspirate after incubation.
13. Add 25 µL Streptavidin-Phycoerythrin to each well containing the 25 µL of Detection Antibodies.
14. Seal, cover with foil, and incubate with agitation on a plate shaker for 30 minutes at room temperature (20~25°C).
15. Gently remove well contents and wash plate 2x following instructions listed in the Plate Washing section.
16. Add 150 µL of Sheath Fluid to all wells, and resuspend the beads on a plate shaker for 5 minutes. l and 75 beads per bead set.µ
17. Run plate on Bioplex 200, and set parameters for 125
18. Save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method for calculating cytokine/chemokines concentrations in samples. For diluted samples, multiply the calculated concentration by the dilution factor.
19. Plate Washing:
    a. Let plate "soak" on magnet for 60 seconds to allow complete settling of the magnetic beads.
    b. Remove well contents by aspiration.
    c. Wash plate with 200 µL/well of Wash Buffer
    d. Allow beads to soak for 60 seconds, and remove Wash Buffer by aspiration after each wash.
    e. Repeat wash steps as recommended in the Assay Procedure.
    f. If using the recommended plate washer for magnetic beads (Bio-Tek ELx405), follow the instructed equipment settings. Mouse Metabolic Phenotyping Centers MMP

8.8 APPENDIX 8: SOPS FOR IMMUNO-HISTCHEMICAL STUDIES

1. Keloids specimens will be cut into 4um specimens

2. Specimens will be deparaffined and hydrated

3. Specimens will then be incubated in 3 %hydrogen peroxide in distilled water for 5 minutes

4. Specimensincubated with primary antibody and diluted 1 /75 for 10 to 30 minutes

5. Specimens will then be incubated with a biotinylated link antibody and peroxidise –labelled streptavidin

6. Specimens antibodies will be completed by incubation of sections with the chromogenic substrats solution 3-3' Diaminobenzidine for 10 minutes

7. Specimens will be counterstained with Meyer's haematoxylin for 1 minute followed by cleaning with tap water

8. Dehydration of the specimens will be done through a graded ethanol series 70%, 96%, 100 % for 3 minutes

9. Clearing of the sections though toluene and eukit

10. Semiquantitave assesment of the specimens will be done.

APPENDIX 9: Publications from the study

8.9.1: Nangole FW, Agak George; Keloids pathophysiology, fibroblasts or inflammatory disorder

***JPRAS Open*Volume 22, December 2019, Pages 44-54**

ABSTRACT

Background: Keloids are defined as a benign dermal fibroproliferative disorder with no malignant potential. They tend to occur following trivial trauma or any form of trauma in genetically predisposed individuals. Keloids are known to grow beyond the margins of the wound and are common in certain body parts. The pathophysiology of keloid remains unclear, and fibroblasts have been presumed to be the main cells involved in keloid formation. Understanding the mechanism(s) of keloid formation could be critical in the identification of novel therapeutic regimen for the treatment of the keloids.

Objective: To review the pertinent literature and provide updated information on keloid pathophysiology.

Data Source: A Medline PubMed literature search was performed for relevant publications.

Results: A total of 66 publications were retrieved, with relevant publications on the etiology and pathogenesis as well as experimental studies on keloids. All articles were critically analyzed, and all the findings were edited and summarized

Conclusion: There is still no consensus as to what is the main driving cell to keloid formation. One may, however, hypothesize that keloid formation could be a result of an abnormal response to tissue injury, hence resulting in an exaggerated inflammatory state characterized by entry of excessive inflammatory cells into the wound, including macrophages, lymphocytes, and mast cells. These cells seem to release cytokines including transforming growth factor β1 that stimulate fibroblasts to synthesize excess collagen, which is a hallmark of keloid disease.

8.9.2.Ferdinand W Nangole 1, Kelsey Ouyang, Omu Anzala, Julius Ogeng'o, George W Agak, Daniel Zuriel, Does Keloid Histology Influence Recurrence? *Am J Dermatopathol*2021 Jan 12.doi: 10.1097/DAD.0000000000001880

- PMID: 33464754DOI: 10.1097/DAD.0000000000001880

ABSTRACT

Keloids are fibroproliferative disorders characterized by high recurrence rates, with few factors known to influence the same. We conducted a study to determine whether keloid histology influences recurrence. This was a prospective longitudinal study to determine whether histopathological parameters of keloid influence recurrence. Patients with keloids managed by surgical excision were followed up at Kenyatta National Hospital between August 2018 and July 2020. The excised keloids were processed for histology using hematoxylin,/eosin, Masson, and trichrome stains. The slides were analyzed for inflammatory cells, fibroblasts, and capillary density using the hot spot technique and correlated to keloid recurrence. Postoperative follow-up was for a minimum of 1 year. A total of 90 patients with 104 keloids were recruited in the study. Overall keloid recurrence rate was 28.6%. There was a correlation between the absolute count of more than 50 per High power field of lymphocytes, fibroblasts, and macrophages with recurrence of the disease. The sensitivity and specificity for the above parameters were lymphocytes 48% and 81%, macrophages 57% and 83%, mast cells 32% and 33%, and fibroblasts 41% and 91%, respectively. There was no correlation between mast cells and vascularity status with recurrence. Routine histology should, therefore, be performed to determine these parameters. Close monitoring and second-line therapy should be considered for patients with elevated macrophages and/or lymphocytes so as to reduce the risk of recurrence

8.9.3 :Nangole FW, Ouyang K, Anzala O, Ogengo J, Agak GW. Multiple Cytokines Elevated in Patients with Keloids: Is It an Indication of Auto-Inflammatory Disease Journal of Inflamm Res. **2021;14:2465-2470 https://doi.org/10.2147/JIR.S312091**

Background: Inflammation seems to play a major role in the pathophysiology of keloids. However, the role of cytokines in keloid pathophysiology has not been fully evaluated with only a few cytokines studied. We undertook this study to compare various cytokines in patients with keloids and neither a control group of patients without keloids nor family history of keloids so as to determine which cytokines are elevated and could thus be critical in keloid formation. **Methods:** This was a cross-sectional study of patients with keloids and a control group of those without. Patients in both groups were matched for age, sex and body mass index. Their plasma was analyzed for both inflammatory and anti-inflammatory cytokines using the Bio-flex Elisa^{TM} method. Comparisons of cytokines means in both groups were done using Student's *t*-test.

Results: A total of 84 participants with 42 participants in each group were followed during the study. Male to female ratio was 1:2. Age ranges were similar with a mean of 29.6 years. A total of 28 cytokines were assayed. Statistically significant differences were noted in 15 of the 28 cytokines assayed with 11 being elevated more in keloid patients with only four in the non-keloid forming group. Among elevated cytokines in keloid patients were granulocyte colony-stimulating factors, granulocyte-monocyte-colony-stimulating factors, interleukins 4, 6 and 13. **Conclusion:** Patients with keloids have significantly higher cytokines compared with non-keloid forming patients. This finding suggests that keloid formation could be influenced by multiple inflammatory cytokines, an indication that the patient's immune system could play a role in keloid formation akin to auto-inflammatory disease.

Keywords: keloids, cytokines, auto-inflammatory, disease

8.9.4 : Nangole F.Wanjala[1],GithaigaJoseph[1],MamatiAnthony[1],O.Primus[1],James J.Ogeng'o[2],OmuAnzala[3],IsabellaDohil[4],George WAgak[4]
Keloids: Does Patients Sex Influence the Presentation and Recurrence post excision
J Plast Reconstr Aesthet Surg. 2022 Jan;75(1):366-368. doi: 10.1016/j.bjps.2021.08.030. Epub 2021 Sep 20. PMID: 34642061.

8.9.5 : Blood group and Human Leucocyte Antigen sub-type as determinants to keloid formation and Recurrence in Keloid Patients; A prospective longitudinal cohort study J **Dermatol Res. 2021;2(3):1-10. DOI: http://dx.doi.org/10.46889/JDR.2021.2302**
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Abstract

The role of genetic factors in keloid is affirmed by the fact that keloids have been shown to occur among members of the same family. We undertook this study to determine whether there is any association between patients' blood group and HLA sub-types to keloids and keloid recurrence.

This was a prospective longitudinal study of patients with keloids and a control of patients managed for other surgical conditions with no keloids. Blood was taken from each patient and analyzed for blood group and HLA sub-types using the sequence specific primer geno-typing. Data captured were summarized and analyzed using students T-test and Bonferroni correction. Probability values significance was at 0.05.

A total of 90 patients with keloids and 59 in a control group were followed up in the study. The male to female ratio of the patients was 2:1. The most common blood group for both groups was blood group O at 51.3% and 49.2%, followed by blood group A and B respectively. Patients with keloids had a significantly higher positive alleles of HLADQA1*01 and HLADQB1*06. There was also an association between blood group A andkeloid recurrence.

In conclusion, this study demonstrates that there is significant difference in some HLA sub-types and blood groups among patients who form keloids and non-keloid forming patients an indication of the possible role patients genetics and immune could play in keloid pathogenesis and severity.

Key words: Blood group, Human Leucocyte Antigen, keloid, Recurrence

8.9.6 Manuscript Title: Treatment outcomes of extra-lesional and intra-lesional of keloid excision followed by post-excision superficial radiotherapy: A comparative study;

Ferdinand W. Nang'ole, [1] Kelsey Ouyang, [2] Omu Anzala, [3] Julius Ogeng'o, [4] and George W. Agak, [2]

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ABSTRACT

Background:. Surgical management of keloids has been performed by either intralesional or extralesional excision. There has been no consensus regarding the optimal treatment for reducing recurrence and complications. We undertook this study to compare the outcomes of patients managed with intralesional or extralesional excision followed by post excision radiotherapy.

Objective:This was a comparative study to determine the outcome of keloids managed with intralesional or extralesional excision followed by postexcision superficial radiotherapy.

Results: A total of 90 patients with 104 keloids were managed during a two and half years prospective study, with 56 keloids operated on in the intralesional group (IL) and 58 keloids operated on in the extralesional (EL) group. The postsurgery pruritus score after two year of follow-up was 1.98 in the IL group and 1.86 in the EL group (p value>0.05) . Pain score was at 2.8 and 1.129 in the EL and IL respectively (*P*-value >0.05). The recurrence rate was 21% in the IL group and 18.9% in the EL group (*P*-value >0.05). Overall patient's satisfaction was good in either group.

Conclusion: There was no difference in the outcome of keloid excision between the two treatment modalities. We concluded that both modalities can be utilized in managing keloids. Further research should be done to compare both treatment methods with other adjunct therapies, such as steroids and cytotoxic agents.

Key words: Keloids excision intralesional extralesional recurrence

Submitted to the Archives of Plastic Surgery, Manuscript ID, 21-119

8.9.7 : Ferdinand W. Nangole, [1] Kelsey Ouyang, [2]Omu Anzala, [3] Julius Ongengo, [4] George W Agak[2]
Familial and Sporadic keloids: A comparative study on Presentation, Management and Outcome
East African Medical Journal; 2022;99;(5) ;69-74

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Abstracts

Introduction

Keloids are dermato-fibro-proliferative disorders characterized by excessive collagen deposition in the dermal layer. While most keloids occur sporadically, a genetic predisposition is supported by both familial aggregation of some keloids and the large differences in risk among populations. We carried out a comparative study to determine the presentation and management outcome of patients within the familial and sporadic keloid groups.

Objective: To determine the clinical presentation and outcomes of familial and sporadic keloids among patients at Kenyatta National Hospital.

Patients and Methods

This was a longitudinal prospective comparative study of patients who presented with keloids at Kenyatta National Hospital. Patients with keloids were classified into familial or sporadic groups based on the family history of the disease. Patients in both groups were managed by surgical excision followed by superficial radiotherapy. They were then followed up for at least one year to determine the recurrence of the disease.

Results: A total of 90 patients with 121 keloids were seen. The male to female ratio for the sporadic group was 1:3 while the ratio was 1:1 for the familial group ($p<0.001$). The age of onset for the familial keloids was 17.8 years and 18.5 years for the sporadic keloids. The most common anatomical site for keloids in both groups were the ears with the second most common site to be the cheeks for the familial group and the abdomen for the sporadic group ($p<0.05$). 60% of familial keloids presented with pain compared to 42% of the sporadic group. 98% of

familial keloids presented with pruritus compared to 61% of sporadic keloids (p<0.001). The recurrence rate was 26.9% for familial keloids compared to 18.5% for the sporadic keloid group (p< 0.026).

Conclusion: This study demonstrates that patients with familial keloids have a high pain and pruritus prevalence as well as a high recurrence rate, and therefore a more severe disease in comparison to the sporadic keloids patients. This suggests that familial keloid patients need a more aggressive management strategy compared to the sporadic group.

Key words: Keloids; Familial; Sporadic; Recurrence

Submitted to the European Journal of Dermatology

8.9.8 : Manuscript title: Mesenchymal Stem Cells in Normal Skin, Keloids and Keloids with Recurrence

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Abstract

Purpose: To determine Mesenchymal stem cells (MSC) in keloids and normal skin as a determinant of keloid recurrence.

Patients and Methods: This was a longitudinal prospective study in which patients with keloid excision had their specimen analyzed for MSC. A control group of patients matched for age, sex

and Body Mass Index (BMI) with no history of keloids admitted for elective surgical procedures had their skin samples taken and also analyzed for MSC. Data collected was analyzed using student T test and Chi square test to compare means and standard deviations

Results: A total of 61 patients with keloids and a control group of 32 were recruited. The male female ratio for the patients was 1:2 with a mean age of 29.5 and 29.7 for the keloids and the control respectively. Patients with Keloids with recurrence had a mean density of 841.4 MSC/gram compared to 578 MSC/gram of tissue for those with no recurrence and 580 MSC/gram for patients with normal skin. Recurrent keloids had a significantly higher percentage of MSC compared to those without.

Conclusion: Keloids compared to the normal skin had a higher percentage of MSC with recurrent keloids demonstrating even higher counts, a possible indicator that MSC correlates with severity of keloid disease.

Key Words: Stem cells, Skin, Keloids, Recurrence. **Submitted to the Journal of Burn ; ref no JBUR-D-21-00644**

8.9.9. Manuscript title: Determinants of keloid recurrence to Patients at Kenyatta National Hospital: The role of keloid recurrence scoring system;

Nangole F.W., Anzala O, Ogeng'o J, Agak G

1

ABSTRACT

Background: Keloid disease is a fibro-proliferative disorder characterized by excessive deposition of collagen. Keloids have been shown to have high recurrence rate. We undertook this study to determine what factors could influence recurrence of the disease with the aim of developing a keloid recurrence scoring system.

Methods: This was a prospective longitudinal study of patients who presented with keloids, managed by surgical excision followed by post excision radiotherapy. Post-surgery patients were followed up for at least two years to determine recurrence. Variables analyzed included patients history, clinical presentation and keloid histology. Data captured were analyzed using SPSS

version 21. Student T-test and Chi-square test were used to compare means and frequencies respectively at 95 percent confidence level (*P*-Value <0.05).

Results: Ninety patients were followed up in the study for a minimum duration of two years. Overall keloid recurrence was 21 % with Male patients having a significantly higher recurrence rate of 31% compared to the females at 12%. The recurrence rates were also higher in familial keloids at 26.9 % compared to sporadic keloids at 18.5%. Other factors that influenced recurrence included anatomical location, patient's blood group and histological composition of the keloid.

Conclusion: Keloid recurrence is influenced by many factors including family history, clinical presentation and keloid histology. A Keloid recurrence scoring system encompassing these factors could assist in the determination of post excision management as well as prediction of the likelihood of recurrence.

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