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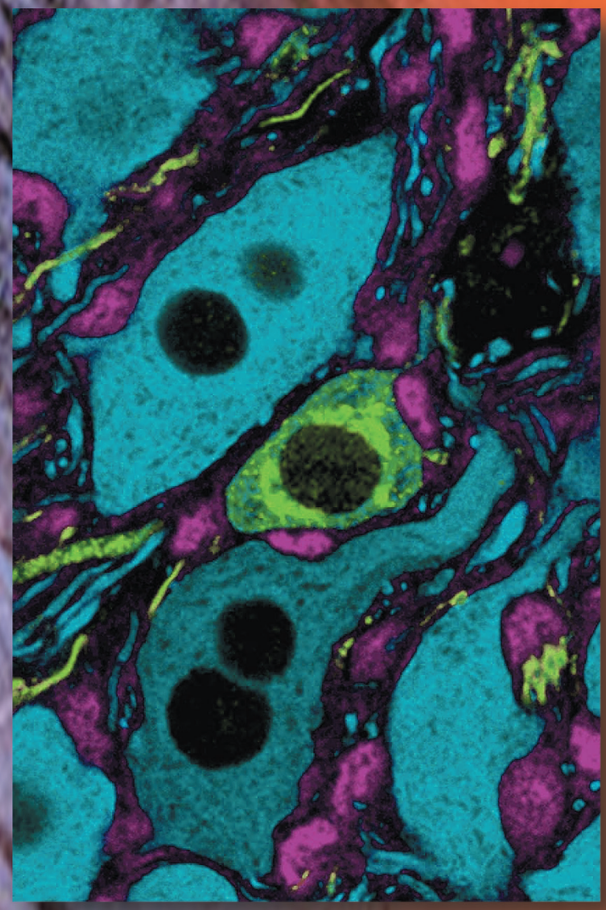

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ABO blood group secretor status correlates with autoimmune diseases in Myanmar patients

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Abstract

In the ABH blood group system secretors release H, A or B antigens into body secretions, the mechanism of which is governed by the fructosyltransferase 2 (*FUT2*) gene. The objective of our study was to elucidate whether ABH non-secretors have an increased tendency for autoimmune diseases. A cross sectional laboratory-based 1:1 case-control study was performed among already-diagnosed autoimmune disease patients and normal blood donors.

In this study, 29% of the patients and 16% of the controls were non-secretors with the numbers of non-secretors higher in every blood group compared to the control group. Patients were suffering a variety of autoimmune disorders: systemic lupus erythematosus (SLE) (51%), rheumatoid arthritis (RA) (26%), progressive systemic sclerosis (14%), mixed connective tissue diseases (5%), overlapping syndrome (2%), immune vasculitis (1%) and Takaya syndrome (1%). This study found that 29% of SLE patients and 27% of RA patients were non-secretors resulting in a statistically significant higher proportion of non-secretors in individuals with autoimmune conditions ($p = 0.028$).

Keywords: *ABO blood group, secretor status, autoimmune diseases, systemic lupus erythematosus, rheumatoid arthritis*

Introduction

In the ABH blood group system, non-secretors do not produce H, A or B antigens while secretors release these antigens via a mechanism governed by the *FUT2* gene (Daniels 2013a). The genotype of secretors is Se/Se or Se/se. Secretors secrete the H antigen which is processed into A and/or B antigens depending on their ABO genotype. Non-secretors are homozygous for se (se/se). They cannot produce a soluble form of H antigen and hence do not produce A and B antigens (Dean 2005).

The *FUT2* gene (Se locus) is located on chromosome 19 at 19q13.3. It has two exons which is about 25 kb of genomic DNA. The enzyme fucosyltransferase is encoded on the Se locus and presented in the epithelia of secretory tissues like salivary glands, the gastrointestinal tract and the respiratory tract. It catalyzes Type-1 and Type-3 glycan to form the H antigen in bodily secretions (Stanley and Cummings 2017).

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FUT2 encodes a 332 amino acid polypeptide. The common non-secretor allele of *FUT2* in people of European and African origin is *se-428* (428G>A nonsense mutation) (Gly247Ser substitution) which causes no active enzyme production. Another *FUT2* allele (*Se-w385*) (Ile129Phe substitution) is common in Eastern Asia and the South Pacific. Although this enzyme has identical substrate specificities to the normal *FUT2* product, it has at least a five-fold reduction in enzyme activity. A single, multiplex PCR technique followed by restriction fragment length polymorphism digestion has been devised to detect many of the known *FUT2* mutations (Daniels 2013b).

The simplest method for determining secretor status is by inhibition of haemagglutination. Secretors have blood group antigen in their saliva which can react with diluted antisera and no agglutination can be seen when red blood cells expressing the apprpriate antigens are added. Conversely the presence of agglutination indicates a non-secretor.

About 80 % of individuals are secretors and they secrete ABH antigens into their body secretions except for cerebrospinal fluid. ABH non-secretors are prone to suffer from autoimmune diseases such as Sjögren's syndrome, multiple sclerosis, ankylosing spondylitis, reactive arthritis, psoriatic arthropathy and Grave's disease (D'Adamo and Kelly 2001).

Studies have shown that non-secretor status has a statistically significant association with some autoimmune diseases. For instance, Dickey and colleagues in 1994 found that the non-secretor state is significantly associated with coeliac disease (Dickey *et al* 1994). Another study showed that as the expression of Lewis antigen is correlated with the ABH antigen secretor status, Lewis b-negative non-secretor-individuals are more susceptible to pemphigus vulgaris (Shahidi-Dadras and Golfeshan 2016). Therefore, we would like to determine the ABO blood group secretor status of patients with autoimmune diseases compared to a control group in the Myanmar population.

Materials and methods

Study population

A 1:1 ABO blood group matched case-control study was undertaken with autoimmune patients and healthy blood donors from January 2018 to January 2019. Already diagnosed systemic autoimmune diseases patients, who visited as out-patients or attended at the Department of Rheumatology, Yangon Special Hospital in Myanmar were randomly selected and they volunteered to participate in this study. Whole blood and saliva were collected for blood group and ABO blood group secretor status determination. Regular blood donors who had consented according to the blood donor selection process were selected as the control group. Sample size was calculated as follows:

$n = (r+1)/r * (p)(1-p)(Z\beta+Z\alpha/2)2 / (P1-P2)2$

r = ratio of control to case

p = expected true proportion

P1 = proportion of case exposed = Odds Ratio * P control exp / P control exp * (Odds Ratio -1)+1

P2 = proportion of control exposed

Odds ratio = 2.5 (minimum odds ratio to detect in secretor-status and autoimmune disease) (Dickey *et al* 1994)

Exposed controls = 25% (percentage exposed among controls) (Bharath and Arumugam 2016)

One-sided alpha risk = 5%, $\alpha = 0.05$

Power = 90%, $\beta = 0.1$

Controls / case ratio = 1

Total exposed = 35.2273%

Estimated sample size:

Number of cases = 92

Number of controls = 92

By adding 10% attrition rate, minimum numbers of cases and control were 100 each.

ABO blood grouping

ABO blood grouping of participants was performed by the tube method. Equal volumes of monoclonal anti-A and anti-B grouping reagents (Swemed Diagnostics, Bengalura, India) and 2% cell suspensions of participant's red cells were used for forward grouping. Equal volume of participant's serum and 2% suspensions of known A and B red cells were used for reverse grouping. The serum samples were also tested with the participant's own cells and group O cells to exclude reactions other than due to anti-A or anti-B in the sample. The suspensions were mixed by tapping the tubes and leaving undisturbed for 15 min before centrifugation for 1 min at 112 g. The tubes were then examined for any agglutination (Rowley and Milkins 2006).

Test for ABH substance secretion

Anti-A, anti-B (IgM/Swemed Diagnostics) and anti-H (extract from *Ulex europaeus* seed, National Blood Centre, Thai Red Cross Society, Bangkok, Thailand) were used. The antisera were serially diluted and the last dilution giving a 2++ reaction with corresponding red cells was used in the inhibition studies. Several milliliters of saliva were placed in the tube, incubated for 30 min at 56° C and then centrifuged. Six tubes labelled as 'A', 'B', 'H', 'A-Control', 'B-Control' and 'H-Control' were set up. One volume of supernatant was added into each tube labeled as 'A', 'B' and 'H'. One volume of saline was added in each of the three control tubes. One volume of the diluted anti-A was added into each 'A' and 'A-Control' tubes, anti-B into each 'B' and 'B-Control' tubes and anti-H into each 'H' and 'H-Control' tubes. After mixing the content of tubes, they were incubated at 37°C for 30 min. One volume of 2% suspension of group A cells into each 'A' and 'A-Control' tubes, group B cells into each 'B' and 'B-Control' tubes, and group O cells into each 'H' and 'H-Control' tubes were added. The contents were mixed and incubated at 37°C for 30 min and inspected for agglutination. A range of temperatures (4°C, 20°C, 25°C and 37°C) for agglutination were also tested and the result at 37°C was found satisfactory for ABH substances. If the saliva contains A, B or H substances, agglutination was inhibited in the 'A', 'B' or 'H' tubes respectively except the control tubes (Knowles and Regan 2006).

In our study, we used undiluted saliva. Although saliva is hypotonic, the agglutination result was interpreted satisfactorily. This may be due to the saliva being mixed with antisera and 2% red cells suspension which are diluted with normal saline. The interpretation of red cells agglutination was also carried out immediately. The agglutination results were read by at least two researchers and the results were matched and confirmed by another

researcher.

Statistical analysis

Data entry and statistical analysis were performed using Statistical Package for Social Science Software, version 20.0. The association was calculated by means of chi-square and a 'p' value less than 0.05 was assumed as statistically significance.

Ethical consideration

This study was approved by the Institutional review board of the University of Medical Technology, Yangon, Myanmar (IRB No 2/2018-4).

Results

Demographic data

In this study, 39%, 34%, 23% and 4% of the patient and control group were blood group B, O, A and AB respectively. Mean age of the patient group is 36.8 years (SD=15.5 years) and that of control group is 31.7 years (SD=10.5 years).

In this study, the female proportion is higher in both groups (94% in patient and 76% in control group). It has been recognised that autoimmue diseases are more common in female as females have increased immunoreactivity compared to males (Zandman-Goddard *et al* 2007).

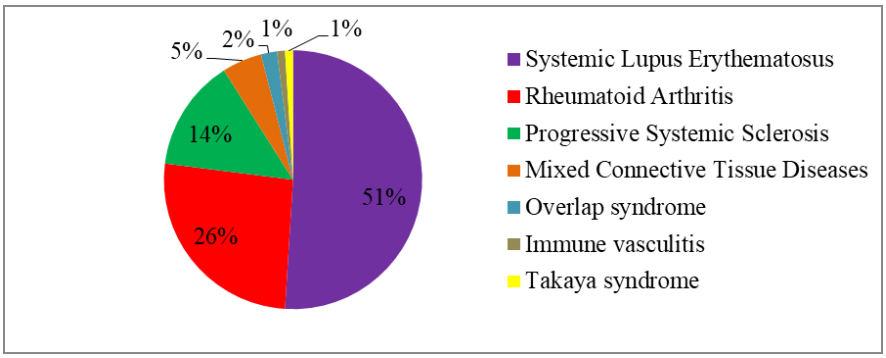

Figure 1. *Range of autoimmune diseases in our patient group.*

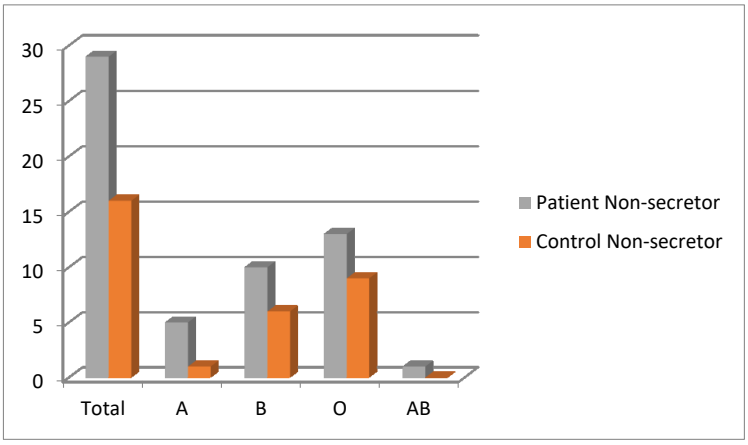

Figure 2. *Numbers of non-secretors for each blood group.*

Autoimmune diseases and secretor status

The range of autoimmune diseases in our patient group is shown in Figure 1. In the patient group, 29% were non-secretors, while in the control group 16% were non-secretors. The proportion of non-secretors is significantly higher in the patient group than in the control group (p=0.028). Numbers of non-secretors were higher in the patient group in every blood group compared to the control group (Figure 2). The majority of patients in this study suffered from SLE (51%) and RA (26%). The data shows that 29% of SLE patients and 27 % of RA patients were non-secretors.

Discussion

There was a statistically significant higher proportion of non-secretors in individuals with autoimmune conditions (p = 0.028) in our study showing that ABO blood group non-secretors tend to have a higher incidence of autoimmune diseases compared to the control group.

In the ABO blood group system, A, B and H red cell antigens are a carbohydrate terminal of glycoproteins and glycolipids. They are differentiated by the nature of the immunodominant terminal monosaccharide. H antigen has L-fucose linked to D-galactose in its structure; A and B antigens have N-acetyl-D-galactosamine and D-galactose respectively attached to galactose residue. These carbohydrate structures on cell surfaces can be exploited by pathogenic microorganisms to gain entry to the cell or to facilitate microorganism survival (Daniels 2013a).

The important step in pathogenesis of infection is the close contact between microorganism and host cell. Some bacteria can attach to host cells by afimbrial adhesins which are actually the carbohydrate-binding proteins. Such adhesins can be found in viruses, fungi and protozoa (Kok and Pechère 2004).

In a study by Henry and Samuelsson in 2000, it was shown that susceptibility to infection by numerous pathogenic microorganisms is associated with ABO phenotype or secretor status (Henry and Samuelsson 2000). ABH non-secretors are more susceptible to infections caused by *Haemophilus influenzae, Neisseria meningitides*, and *Streptococcus pneumoniae* and urinary tract infection by *Escherichia coli* than secretors (Anstee 2010). ABH non-secretors are resistant to most Norwalk virus strains (Lindesmith *et al* 2003; Thorven, Grahn *et al* 2005). Some degree of resistance to HIV-1 infection can also be seen in the non-secretor genotype (Anstee 2010). There are also animal models which have molecular mimicry that may be a trigger of autoimmune diseases (Davidson and Diamond 2001). In a study of metabolic and immunologic consequences of ABH secretor, non-secretors were prone to have a variety of autoimmune diseases (D'Adamo and Kelly 2001).

The immune response to invading microorganisms proceeds from innate immunity to adaptive immunity and macrophages can act as the bridge in this process. Macrophages have various receptors which can recognize microbial surface structures and can degrade the microorganisms in phagosomes and then produce the peptides for presentation in the immune response. They may also take up soluble antigens through the process of pinocytosis. They eventually scavenge dead or dying cells, which have many forms of self-antigens (Murphy 2012).

On the other hand, activation of the innate immune response is a feature of autoimmune diseases and this activation may be the first event involved in triggering of the disease (Mills 2011). Infection can initiate the immune activation which can persist in the absence of any detectable microbial antigen (Davidson and Diamond 2001).

Secretor status in SLE and RA

We found 27% of RA patients and 29% of SLE patients were non-secretors. Pathologic effector mechanisms in rheumatoid arthritis assumes that an autoantigen is taken up by antigen presenting cells and causes the activation of the innate and adaptive immune system. One of the suggested causes of RA is the microbiome. It can lead to increased prevalence of periodontitis in RA and there is an association with particular bacteria including *Porphyrymonas gingivalis* (Smolen *et al* 2014). In the pathogenesis of SLE factors such as drugs, diet, and environmental toxins are implicated through epigenetic mechanisms (Ballestar *et al* 2006; Richardson 2009).

Limitation of the study

We used the haemagglutination inhibition method to detect the ABO blood group secretor status. Secretors who have low ABH blood group antigen cannot be detected in our study as we diluted the antisera to a dilution giving a 2++ reaction with corresponding red cells. On the other hand, small quantities of H, A, and B substances can be detected in the saliva of non-secretors due to the action of the enzyme of *FUT1* gene, but they are generally weak and only reactive at low temperatures (Daniels 2013a).

There are also limitations with anti-H reagent non-specificities as murine monoclonal anti-H antibodies are usually not inhibited by secretor saliva as they react only with Type 2 H of red cells, but not with salivary substances. Anti-H can be prepared from lectin extracts such as seeds and eel serum. Anti-H which is specific for fucose and GlcNAc is produced from *Ulex europaeus* (gorse) seed. It is commonly used for detection of H secretion. Anti-H which is specific for monosaccharide GlcNAc is produced

from seeds such as *Cystisus sessifolius*, *Laburnum alpinum*. Anti-H (-HI) which is specific for fucose is produced from *Lotus tetragonolobus* seed or *Anguilla Anguilla* eel serum (Daniels 2013a).

Conclusion

In our study 29% of autoimmune diseases patients were non-secretors compared to 16% of the control group. There was a statistically significant higher proportion of non-secretors in individuals with autoimmune conditions (p=0.028) in this patient group in Myanmar. We suggest that ABO blood group function may contribute to immunity status in autoimmune diseases.

Acnowledgements

We extend our gratitude to the Department of Medical Research, Myanmar for financial support for this study (External grant no. 23/2018). We also appreciate the help from individuals from the Yangon Special Hospitals, National Blood Center, University of Medical Technology, Yangon, Myanmar, and especially, the participants who were willing participants in this study.

Conflict of interests

There is no conflict of interest for this study.

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The clinical features and dermoscopic patterns of cutaneous metastases arising from ovarian teratoma: an unusual case report

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Abstract

Cutaneous metastases are rare clinical manifestations of malignancy with metastasis. Early signs of cutaneous metastasis are firm polymorphic lesions of erythematous papules, nodules or plaques that may be accompanied by pruritus and pain. Early diagnosis of skin metastases can be a predictor in determining prognosis and in some cases even detecting the presence of a hidden primary malignancy. We report a 70-year-old woman with a stage IVb ovarian teratoma. Erythematous nodules and plaques were found on the left femur, abdomen and posterior trunk. Dermoscopic examination showed arborizing blood vessel and histopathologic result showed mitotic cells, eosinophilic cytoplasm and squamous differentiation, supporting a diagnosis of cutaneous metastasis. On the 20th day, the erythematous nodules were observed to increase in number and size. Unfortunately, the administration of chemotherapy could not be given due to the weak general condition of the patient. This case emphasizes the importance of considering cutaneous metastases in patients with history of malignancy who present with erythematous plaques and nodules, especially those located near the location of the primary tumour.

Keywords: cutaneous metastasis, ovarian teratoma, dermoscopy

Introduction

The cutaneous metastases of internal malignancies are uncommon, and the incidence varies between 0.7 to 10 % of patients (Lookingbill *et al* 1990). Tumours with high tendency to spread to the skin tissue may include carcinoma, melanoma, and hematolymphoid malignancies, germinal cell tumours and sarcoma. Cutaneous clinical manifestations may be unspecific, vary widely, and sometimes are misdiagnosed. Numerous tumours do not only show some tendency to metastasize to specific sites, but produce specific patterns near the primary tumour (Oualla *et al* 2012, Chernoff *et al* 2014).

In many cases, the primary site of the malignancy is unknown, thus the detection of the cutaneous metastasis should be done with high clinical suspicion and may include costly diagnostic modalities such as PET-scan. Early diagnosis is important in order to deliver effective and accurate treatments on the primary tumours in which the clinical symptoms have not explicitly identified. Clinical experience as a dermatologist is needed to identify the wide variety of clinical manifestations (Hussein 2010).

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The incidence of cutaneous metastases varies in different populations and previous research has shown that as many as 9% had cutaneous metastases. In a study of 724 patients, Brownstein and Helwig (1972) examine the distribution of skin metastases in both sexes. In men, primary malignancies that most commonly cause skin metastases included lung carcinoma (24%), colorectal (19%), melanoma (13%) and squamous cell carcinoma (12%). In women, the most common etiology included breast cancer (69%), colorectal cancer (9%), melanoma (5%) and ovarian carcinoma (4%) (Brownstein and Helwig 1972). The anterior trunk is the site most commonly affected, whereas the inferior limb is less frequently involved. In men, about 75% of metastatic skin lesions are observed in the facial site and anterior coli, while 75% of cases in women are seen in the anterior trunk. The posterior trunk is a rare location for metastasis. Out of 77 reported cases (75 men and 2 women), skin metastases originated from other sites of malignancies, which included the lungs (28.6%), melanoma (18.2%), gastrointestinal system (14.2%), genitourinary system (10.4%), head and neck (9.1%), haematology (5.2%), breast (5.2%) and other sites (<2%). Metastasis was the main clinical appearance

of malignancy in 7.8% of cases. Skin metastases involve the head and neck (28%), trunk (40%), extremities (18%) and other sites (14%) with an age range of 37-86 years, with an average of 62 years (Sariya *et al* 2007).

We report a 70-year-old female with ovarian teratoma with a suspected cutaneous metastasis manifesting as multiple erythematous nodules and erythematous plaques.

Case report

A 70-year-old female presented to our department with a chief complaint of multiple red nodules on the left inguinal fold that extended to the left thigh, left side of the abdomen, and left posterior trunk in the last five months before admission. The nodules increased in number and size which were accompanied with pain and mild itching. In addition, the skin surrounding the lesions was hard on palpation. History of past illness was significant for ovarian teratoma stage IVb. She had undergone total hysterectomy with bilateral salpingectomy and the result of histopathological examination was consistent with malignant ovarian teratoma. The patient was previously treated with topical antibiotics and intravenous ceftriaxone (2 grams/24 hours), Heparin (1000 IU/hour), Ardium (1 tablet/8 hours followed with 8 IU/24 hours subcutaneous), subcutaneous Novorapid 10 IU/8 hours, and oral warfarin (2 mg/24 hours) for presumed diagnosis of deep vein thrombosis and cellulitis.

Physical examination showed the patient was in good general condition with normal vital signs. Dermatologic examination showed multiple hemorrhagic nodules and plaques on the left inguinal fold, left thigh, left side of the abdomen, and left side of the posterior trunk with sclerotic surrounding area (Figure 1).

Laboratory examination showed anemia 10 mg/dl, hyperglycemia 233 mg/dl, hypoalbuminemia 2.5 g/dl, and leukocytosis 15,410/ul. Dermoscopic findings revealed extensive arborizing blood vessel appearance (Figure 2). Histopathologic examination of the nodule showed normal epidermal layer and clusters of malignant cells infiltrating the dermal layer with atypical nucleus and pleomorphic, hyperchromatic and prominent nucleoli (Figure 3). These results suggested glandular epithelium origin which partially showed squamous differentiation, suggesting a metastatic adenocarcinoma originating from the ovarian teratoma.

After confirming a diagnosis of cutaneous metastasis of ovarian teratoma, the patient was referred to the obstetrics and gynecologic department and chemotherapy was planned after the general condition and albumin level normalized. Unfortunately, the patient passed away three months afterwards.

Discussion

In the case we described above, the clinical manifestations included erythema, multiple nodules, and sclerotic area on the left femoral and gluteal region which first occurred five months earlier and spread to adjacent area. Studies have documented skin metastases which present from asymptomatic dermal/subcutaneous nodules, plaques, papules, tumours, macules, bullous, or papulosquamous lesions associated with pain and tenderness (Wong *et al* 2013). Early diagnosis can determine the prognosis of the disease and appropriate treatment based on the type of the malignancy. Lesions may be single or multiple and usually manifest into papules, plaques or ulcers that can develop into inflammatory lesions over a period of time. Patients with a history of malignancy are at a higher risk

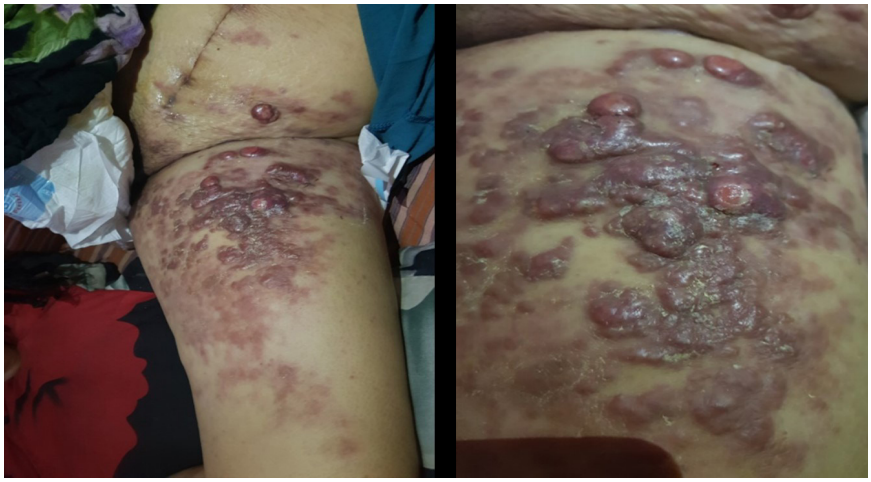

Figure 1. *Multiple nodules, erythematous plaque, and sclerotic area on the left femoral region (left) Close-up picture of erythematus nodules on left thigh (right).*

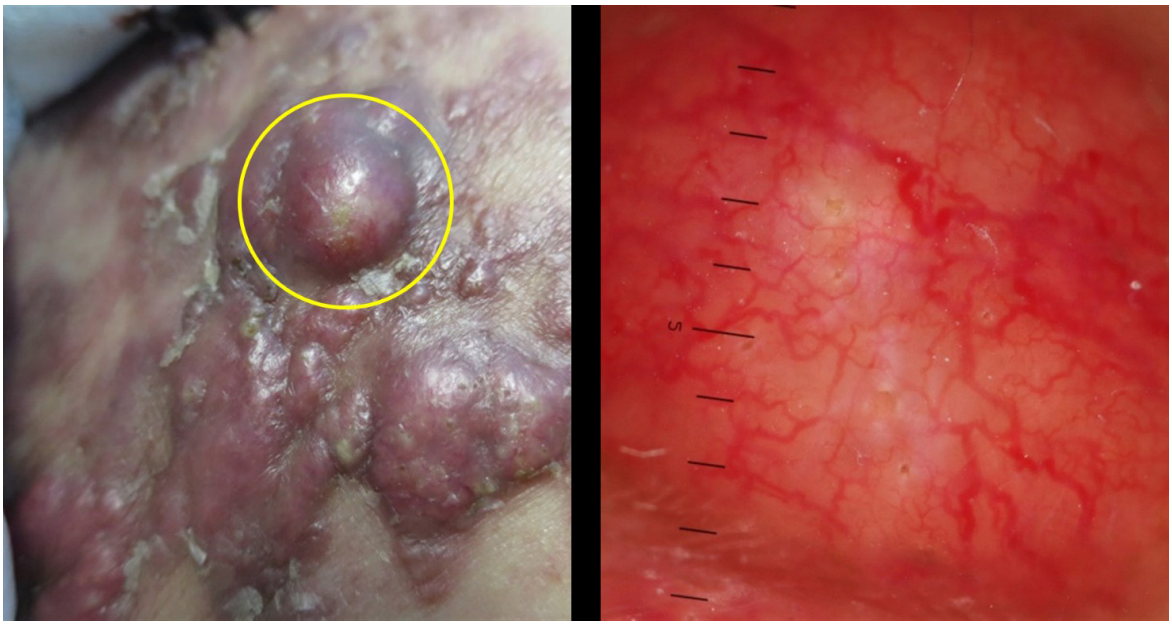

Figure 2. *Dermoscopic finding of a nodule on the left thigh (left) revealed arborizing blood vessels appearance (right).*

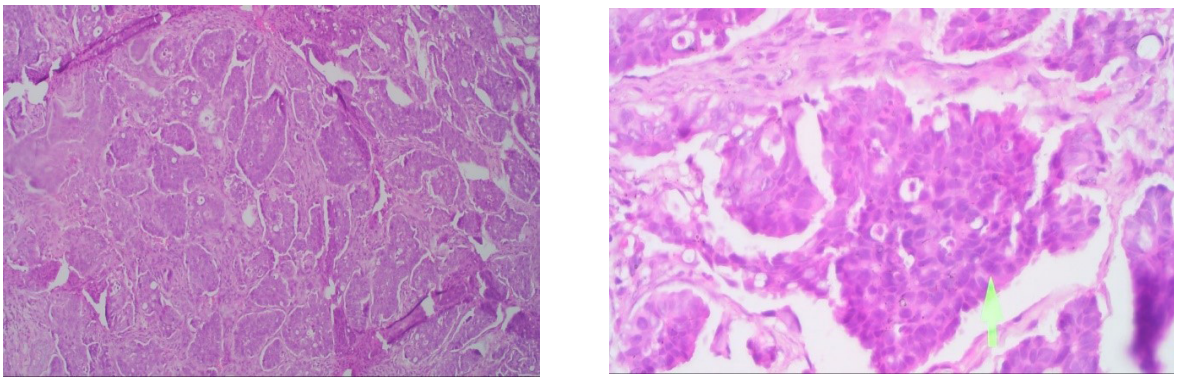

Figure 3. *Histopathological finding showed (A) clusters of malignant cells with squamous differentiation (H&Eosin stain 10x magnification) (B) Atypical nucleus, pleomorphic, hyperchromatin and prominent nucleoli (H&E stain, 40x magnification).*

of cutaneous metastasis in adjacent areas of the primary tumour and can be used to diagnose primary tumours (Sariya *et al* 2007; Gül *et al* 2007). Manifestations in this patient were similar to that described in the literature namely multiple erythematous nodules, erythematous plaques and sclerotic area adjacent to the ovary.

There are several theories that explain the pathogenesis of skin metastases in ovarian cancer, such as the associated lymphatic vessels for spreading (Feki *et al* 2009) however metastasis plays a crucial role in promoting ovarian tumour progression and decreasing patient survival rate (Yeung *et al* 2015). Some patients have cutaneous metastases in surgical scars (laparotomy surgical scars, scarring due to drainage and catheterization actions) (Girijala *et al* 2018; Cormio *et al* 2003). Drainage of the lymph vessels is the most important pathomechanism (Shayan *et al* 2006). The lymphatic drainage is the connection between the periumbilical skin and inner lymph tissue, namely the paraaortic node, the internal mammary node, and the external iliac nodes due to the presence of superficial lymph tissue, especially the axillary and inguinal nodes. In addition, the complex venous meshwork in abdominal wall that drains blood from many places also plays an important role in tumour metastasis (Dubreuil *et al* 1998).

Clinical manifestations of skin metastases can be divided into three types: nodular, inflammatory and fibrotic (brushrial) or sclerodermoid (Cheng *et al* 2017). The most common metastatic lesion occurs as subcutaneous nodules or tumours that are different from the primary skin lesion. It has been found that in cases of cutaneous metastasis lessions may resemble to benign cysts, keratoacanthomas, basal cell carcinomas, or melanomas, especially when there is only single lesion (Sariya *et al* 2007). Skin lesions are usually discrete, oval and round shaped plaques or nodules that are several millimeters to several centimeters in size. Skin metastases are usually bright red to purple in color and can be multiple or solitary and locally or diffusely distributed (Rueben *et al* 2009). Skin biopsies should be performed in cases of suspicious atypical lesions, especially those with solid consistency and multiple in numbers, or accompanied with history of visceral malignancy. Skin metastasis may be correlated with late stage malignancy and sometimes may act as early signs of recurrence (Rueben *et al* 2009). The median time interval between diagnosis of ovarian cancer and skin metastasis is 12 months (1-41 months) (Dauplat *et al* 1987) and patients may be expected to survive up to four months (2-65 months) (Cormio *et al* 2003). The most important prognostic factors which related with survivability is time from diagnosis of ovarian cancer to the occurrence of skin metastasis (Cormio *et al* 2003). Data showed that survival rate is better (mean of 9.7 months) in patients in whom umbilical metastasis were detected before the detection of primary tumours rather than those detected after the detection of primary tumour (Chen *et al* 1998).

Metastasis in the nasal area is reported to be an early sign of ovarian carcinoma, but metastasis can also appear in the chest, back, vulvovaginal area, arms and both legs. These lesions may appear after surgery and grow along with the primary tumours. Some patterns of metastasis have been identified as multiple cutaneous nodules, inflammatory lesions, cicatricial plaques with indurations, macule, infiltrates, discoid lesions, nodules with telangiectasia, and bullous or papulosquamous lesion (Cheng *et al* 2017). Other reported cutaneous manifestations include erysipeloid and sclerotic lesions (Bittencourt *et al* 2015). Cormio *et al* (2003) examined 162 patients with ovarian carcinoma and concluded that significant risk factors for the development of skin metastasis are tumour stage, grades, and the involvements of the lymphoid nodules (Alcaraz *et al* 2012; Liu *et al* 2019). These findings are in accordance with this case, which showed solid multiple lesions at the abdomen, left femoral and gluteal area, with history of ovarian teratoma stage IVb.

Patients with high risk of cutaneous metastasis are those who already have a history of previous malignancies. It has been proven that a single nodular metastatic lesion is often misdiagnosed as a simple cyst or benign connective tissue lesion. The presence of multiple lesions in the same anatomic region and a clinical history of systemic malignancy aid in diagnosing cutaneous metastases. Some case reports show lesions such as pyogenic granulomas, granular cell tumours, benign cysts and angiosarcomas may mimic skin metastases (Gül *et al* 2007; Schonmann *et al* 2003). Primary tumours that are most often accompanied by cutaneous metastases are melanoma, breast malignancy, malignancies of the visceral organs, and squamous cell carcinoma. These cutaneous manifestations can be a measure of poor prognostic factors (Girijala *et al* 2018; Cormio *et al* 2003). The diagnosis for metastatic tumours must also consider the history, histopathological examination, and dermoscopic findings. Differentiation of primary tumours and skin metastases can be distinguished based on histopathological features, especially the type of adenocarcinoma. The neoplastic cells usually line up between collagen collections. In most cases, metastatic lesions may show histopathological features that resemble underlying primary malignancies. However, metastatic lesions usually show anaplastic, pleomorphic cells with hyperchromatin and prominent nucleoli which may also show neoplastic differentiation towards squamous types (Sariya *et al* 2007). The cutaneous metastases can have an extremely wide clinical manifestation and pathologists must have the high clinical-pathologic suspicion to diagnose cutaneous metastases (Habermehl and Ko 2019).

During a dermoscopic examination, we found arborizing blood vessels with pink surface. Patients with skin metastases typically show arborizing blood vessels that are dermoscopically defined as telangiectasias with distinct tree-like branching which was present in this case. This feature may also be seen in vascular tumour, basal cell carcinoma, primary cutaneous malignancy, and various skin disorders (Chernoff *et al* 2014; Liu *et al* 2019). Dermoscopic examination is considered to be an important diagnostic modality with 89% sensitivity and 96% specificity (Mendes *et al* 2013).

Early diagnosis is essential to avoid prolonged anti-inflammatory therapy which will cause a delay in diagnosis (Sariya *et al* 2007). Differential diagnosis of vascular tumours or hemangiomas should also be considered if the lesions bleed easily and quickly enlarge. Pyogenic granulomas usually present as erythematous nodules and are normally found on the face, trunk and extremities. Research shows that cutaneous metastasis can be diagnosed with histopathological examination (Sariya *et al* 2007). In this case, the patient was histopathologically examined because of suspicion towards malignancy due to the increasing number of atypical nodules and sclerotic lesions of unknown cause.

The main treatment of localized cutaneous metastasis is surgical resection while evaluating the general condition and life expectancy of the patient. In extensive cutaneous metastases, palliative therapy is the main treatment due to clinical deterioration that often contraindicates the patient from having chemotherapy (Dauplat *et al* 1987; Liu *et al* 2019).

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SAAL-FOLEY LECTURE

A meander through malaria

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Abstract

Malaria is one of the most well-known and long-established infections in many countries throughout the world. It has contributed to the morbidity and mortality of millions over the years and much research has been done to try and reduce the rate of infection, treat it effectively, and to produce a vaccine. This transcript of the Saal-Foley lecture meanders through the history of the infection to modern day, the milestones in the development of knowledge and treatment of the disease, the eradication efforts over the many years, and the ongoing search for an effective and safe vaccine.

Keywords: malaria, lifecycle, treatment, eradication, vaccine

History

Malaria is an ancient disease. References to what was almost certainly malaria occur as early as 2000 BC on clay tablets from Mesopotamia mentioning deadly periodic fevers, and malaria antigen has been detected in Egyptian mummies from as far back as 3200 BC (Miller *et al* 1994).

In 2700 BC, the Chinese medical canon known as the Nei Chin, linked tertian (every third day) and quartan (every fourth day) fevers with enlargement of the spleen (common in malaria infections) and blamed headaches, chills, and fevers that occur in malaria on three demons—one carrying a hammer, another a pail of water, and the third a stove (Bruce-Chwatt 1988).

Egyptian papyri from 1570 BC reference the common symptoms and this is also seen in Hindu texts as far back as the sixth century BC. More specific writings are found in the early Greeks, including Homer in about 850 BC, Empedocles of Agrigentum in about 550 BC, and Hippocrates in about 400 BC (Sherman 1998). All described the characteristic poor health, malarial fevers and enlarged spleens seen in people living in marshy places.

The arrival of malaria in Rome in the first century AD was a turning point in European history. The disease most likely came from the African rain forest and travelled down the Nile to the Mediterranean, then spread east to the Fertile Crescent and north to Greece. Greek traders and colonists brought it to Italy. From there, Roman soldiers and merchants would ultimately carry it as far north as England and Denmark (Karlen 1995).

The word malaria is believed to come from the Italian 'mal'aria' meaning spoiled air and for over 2500 years the idea that malaria fevers were caused by miasmas rising from swamps persisted until the 1676 discovery of bacteria by Antoni van Leeuwenhoek. From van Leeuwenhoek's observations came the realisation that microorganisms are the causes of infectious diseases and malaria was not just a "swamp fever". With the development of the germ theory of infection by Louis Pasteur and Robert Koch in 1878-1879, the search for the cause of malaria intensified.

In 1880 Charles Louis Alphonse Laveran was the first to identify the malaria parasite. Laveran was a French army doctor during the Franco-Prussian War (1870-1871) and he challenged that the disease was restricted to low-lying humid plains and noted that malaria also could occur in temperate zones and that not all tropical areas were plagued by the disease. He predicted "Swamp fevers are due to a germ" (Jarcho 1984). In October 20, 1880, while looking through a crude microscope at the blood of a febrile soldier, he saw crescent-shaped bodies that were nearly transparent except for one small dot of pigment. With subsequent examination of blood from 148 of 192 malaria patients he saw pigment-containing crescents and then ultimately recognized four distinct forms in human blood of the malaria parasite in different stages of its life cycle: the female and male gametocyte, schizont and trophozoite

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stages (Laveran 1978). He was awarded the 1907 Nobel Prize for the discovery.

In 1897/8 Sir Ronald Ross (a Surgeon-Major in the British Indian Medical Service) demonstrated that mosquitoes transmit malaria. He had spent a fruitless year studying the insects but he was studying those incapable of transmitting malaria. On August 20, 1897 he discovered it in *Anopheles* mosquito that had previously fed on an infected patient and he named this day "Mosquito Day". He continued his studies on *Plasmodium relictum*—an avian malaria—and discovered the stages of the malarial parasite and won the Nobel Prize in 1902 for this work (Sherman 1998).

The Italian scientists Giovanni Battista Grassi, Amico Bignami, Giuseppe Bastianelli, Angelo Celli, Camillo Golgi and Ettore Marchiafava demonstrated the developmental stages for human malaria between 1898 and 1900 by confirming that human malaria parasites pass through the same developmental stages in the mosquito as the avian parasites observed by Ross.

Grassi identified *Anopheles maculipennis* as the vector of human disease in the marshy Roman Campagna and transmitted the malaria parasite *Plasmodium vivax* to a healthy human volunteer.

The last question of where the sporozoites inoculated by mosquitoes undergo development in the human host was solved in 1948. Henry Shortt, Cyril Garnham and colleagues at the Ross Institute of the London School of Hygiene and Tropical Medicine detected malaria parasites in the livers of rhesus monkeys infected with a primate malaria species. They subsequently found similar stages in liver biopsy specimens from human volunteers experimentally infected with *P. vivax* and confirmed it for *P. falciparum* (Garnham 1966).

The final stage in the life cycle, the presence of dormant stages in the liver, was conclusively demonstrated in 1982 by Wojciech Krotoski.

A lot of this work was done on animal, reptiles and bird malaria and there are now 24 species identified in birds and many others in primates and rodents. Some of these are *Plasmodium relictum* and *P. gallinaceum* in birds and chickens, *P. cynomolgi*, *P. inui*, *P. knowlesi* and *P. pitheci* in monkeys and *P. berghei*, *P. yoelii*, *P. vinckei* and *P. chabaudi* in mice, rats, hamsters and gerbils.

Treatment

For many years the ground bark of a Peruvian tree had been used to treat malaria.

"*The Peruvian bark of which the Jesuites powder is made, is an excellent thing against all sorts of Agues*" was written by William Slamon in the Synopsis medicinae in 1671. This bark was from a high-altitude tree native to South America and was named *cinchona* after the Spanish Countess of Chinchon as she was treated with it in Peru in the 1600s (Meshnick 1998).

The ground powder had many names - "Jesuit's powder," "Cardinal's powder," or "Peruvian bark" and was highly sought after and valuable.

In 1820 quinine was purified from tree bark by French chemists Joseph Pelletier and Jean Biename Caventou. Quinine was in high demand worldwide as it was an effective treatment for fevers and commercial plantations of the tree had been established in Java. In World War I Java was invaded and the Allies controlled the supply, so the German government commissioned a search for a quinine substitute following Armistice. The Bayer Dye Works' (a sister company to Winthrop in the USA) chemists tested thousands of compounds until they found a possible substitute.

The first promising agent was Plasmochin (pamaquine) in 1926 followed by Atabrine in 1932 but both had unacceptable side effects. In 1934 Hans Andersag synthesised Resochin (chloroquine) which was passed on to Winthrop, and this was followed by Sontochin (3 methyl chloroquine) but not explored further and forgotten until the outbreak of World War II. The supply of quinine was again cut off with the Japanese occupation of Java. When French soldiers raided a supply of German-manufactured Sontochin in Tunis and handed it over to the Americans, Winthrop researchers adjusted it to enhance its efficacy and called their new formulation chloroquine. They then realised it was the same compound as Resochin when comparing the two compounds (Honigsbaum 2002).

Other drugs that have been found to be effective against malaria are sulfadoxine-pyrimethamine, mefloquine and artemisinin. Sulfadoxine-pyrimethamine is a pyrimidine derivative that emerged from the antimalarial pipeline during World War II but resistance quickly developed. Mefloquine development was a collaborative achievement of the U.S. Army Medical Research and Development Command, the World Health Organization (WHO/TDR), and Hoffman-La Roche, Inc. and was one of 120 compounds produced to try and find replacements for quinine. Preclinical trials coincided with the appearance of chloroquine-resistant falciparum malaria in areas of American military concern in Southeast Asia, and South America. Mefloquine's efficacy in preventing falciparum malaria when taken regularly was first reported in 1974 and soon after it also was shown to be a successful treatment agent (Rieckmann *et al* 1974; Trenholme *et al* 1975). Clinical evidence of parasites resistant to mefloquine began

to appear in Asia around the time of the drug's general availability in 1985 (Hoffman *et al* 1985).

The next drug to come on the scene was known for centuries in China. To reduce fever, "*take a handful of sweet wormwood, soak it in a sheng of water, squeeze out the juice and drink it all*" was written by Ge Hang, a Chinese scholar in AD 340. Artemisinin is the antimalarial principle isolated by Chinese scientists in 1972 from *Artemisia annua* (sweet wormwood), better known to Chinese herbalists for more than 2000 years as qing-hao. Extract of qing-hao fed to mice infected with lethal *P. berghei* was as effective as chloroquine and quinine. Testing qing-hao in humans began and findings were published in the Chinese Medical Journal in 1979 (Klayman 1985).

Now artemisinin and other artemether-group drugs are the main line of defence against drug-resistant malaria in many areas of southeast Asia. To date, there have been no reported cases of genetic resistance.

Eradication

To control and hopefully eradicate malaria, measures are needed to eliminate the vector and the parasite, and effective treatment. Chloroquine and DDT emerged as the two principal weapons in the World Health Organization (WHO) ambitious "global eradication" malaria campaign.

Some of the key dates in the timeline for the eradication and treatment of malaria are:

1939 Paul Hermann Muller in Switzerland tests the insecticide DDT. He wins the Nobel Prize for this work in 1948.

1952 Malaria is eliminated in the United States.

1955 WHO launches the Global Malaria Eradication Campaign, which excludes sub-Saharan Africa and is eventually abandoned.

1957 First documented case of resistance to chloroquine is reported.

Chloroquine-resistant *P. falciparum* (CRPF) probably arose *de novo* from four independent geographic locations: the Thai Cambodian border around 1957, Venezuela and the nearby Magdalena Valley of Colombia around 1960 and Port Moresby, Papua New Guinea, in the mid-1970s. In Africa, CRPF was first found in 1978 in non-immune travellers to Kenya and Tanzania spreading next to inland coastal areas and by 1983, to Sudan, Uganda, Zambia, and Malawi. Current evidence suggests that CRPF strains seen in Africa originated in Asia.

1976 William Trager and JB Jensen grow parasites in culture for the first time, opening the way for drug discovery and vaccine research.

1989 The U.S. Food and Drug Administration approves the use of the anti-malaria drug mefloquine hydrochloride, developed by WHO and the U.S. Army Medical Research and Development Command, with Hoffman-LaRoche, Inc. registered as Lariam®.

Clinical evidence of parasites resistant to mefloquine began to appear in Asia around the time of the drug's general availability.

1996 Insecticide-treated bed nets are proven to reduce overall childhood mortality by 20 percent in large, multi-country African study.

1998 Roll Back Malaria Partnership (RBM) launched by WHO, UNICEF, UNDP and World Bank with goal of halving malaria incidence and mortality by 2010.

2002 Genome sequencing of *Anopheles gambiae* (mosquito) and *Plasmodium falciparum* (parasite) completed.

2007 World Malaria Forum convenes in Seattle, hosted by Bill and Melinda Gates Foundation.

2008 The Global Health Group at University of California, San Francisco (USCF) comes forward with the first high-level strategy for the eventual achievement of malaria eradication. This strategy has since been widely adopted.

It is a coordinated, eight-country effort to achieve malaria elimination in four countries in southern Africa (Botswana, Namibia, South Africa, and Swaziland) by 2020 and pave the way for progressive elimination in four additional countries (Angola, Mozambique, Zambia, and Zimbabwe) by 2030. This is to be achieved by these three strategies. :

- Response: timely response, malaria outbreak mapping;
- Surveillance: identify the high-risk areas to improve targeting of interventions;
- Vector control: insecticide-treated bed nets and indoor residual spraying with insecticide to reduce transmission between mosquitoes and humans.

Outcomes

The 2019 WHO report shows that after an unprecedented period of success in global malaria control, progress has stalled. Data from 2015–2018 highlight that no significant

progress in reducing global malaria cases was made in this period.

It is estimated that the number of cases of malaria in 87 countries rose from 233 million in 2000 to 244 million in 2005 but decreased to 225 million in 2009 and increased slightly to 228 million in 2018. The number of deaths due to malaria is estimated to have decreased from 985,000 in 2000 to 781,000 in 2009 and again decreased to 405,000 related deaths in 2018 (Figure 1).

Mortality rate dropped from 0.42% to 0.20% in that timeframe.

Countries that have achieved at least three consecutive years of zero local cases of malaria are eligible to apply for the WHO certification of malaria elimination.

In recent years, 11 countries have been certified by the WHO Director-General as having eliminated malaria: United Arab Emirates (2007), Morocco (2010), Turkmenistan (2010), Armenia (2011), Maldives (2015), Sri Lanka (2016), Kyrgyzstan (2016), Paraguay (2018) and Uzbekistan (2018). In early 2019 Algeria and Argentina joined this group (Figure 2).

The WHO African Region carries a disproportionately high share of the global malaria burden. In 2018, the region was home to 93% of malaria cases and 94% of malaria deaths (Figure 3).

Total funding for malaria control and elimination reached an estimated US$ 2.7 billion in 2018. Contributions from governments of endemic countries amounted to US$ 900 million, representing 30% of total funding (WHO 2019).

Origin and evolution

To understand how to control and potentially eliminate infection and how to develop a vaccine, we need to understand the origin and evolutionary history of *Plasmodium falciparum* and *Plasmodium vivax* which cause more than 95% of all human infections.

It was widely believed until recently that *P. falciparum* had co-evolved with humans over millions of years, while *P. vivax* emerged in south-eastern Asia following the cross-species transmission of a parasite from a macaque. However, with the discovery of many *Plasmodium* spp. in chimpanzees and gorillas these theories have been refuted and instead it has been revealed that both *P. falciparum* and *P. vivax* evolved from parasites infecting wild-living African apes. It is now known that *P. falciparum* resulted from a recent cross-species transmission of a parasite from a gorilla, while *P. vivax* emerged from an ancestral stock of parasites that infected chimpanzees, gorillas and humans in Africa. *Plasmodium vivax* has been virtually eliminated from human populations in sub-Saharan Africa since then with the spread of the protective Duffy-negative mutation (Loy *et al* 2017).

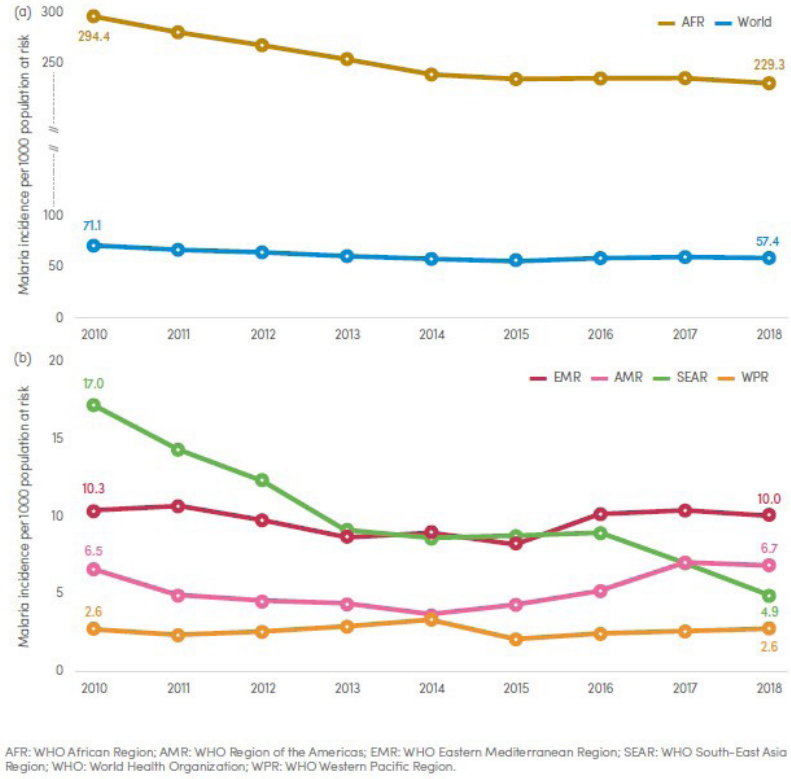
P. ovale- and *P. malariae*-like sequences have been detected in African great apes and additional work is required to ascertain the relationship of these parasites to their human counterparts.

It has been a long complex evolutionary process and many questions still remain concerning the biology and zoonotic potential of the *P. falciparum*- and *P. vivax*-like parasites infecting apes, but comparative genomics, coupled with functional parasite and vector studies, are starting to yield new insights into ape *Plasmodium* transmission and pathogenesis (Liu *et al* 2010).

P. knowlesi, a species that was thought to be a primate malaria only, has also recently been shown to infect humans through the *Anopheles leucosphyrus* group vector in the Malaysian peninsula area (Singh *et al* 2004).

Malaria infection begins when sporozoites, the infective stages, are injected by a mosquito and are carried around the body until they invade liver hepatocytes. They undergo a phase of asexual multiplication (exoerythrocytic schizogony) resulting in the production of many uninucleate merozoites. These merozoites flood out into the blood and invade red blood cells where they initiate a second phase of asexual replication (erythrocytic schizogony) resulting in the production of 8-40 merozoites which invade new red blood cells. This process is repeated almost indefinitely. After usually 10-12 days, some young merozoites develop into male and female gametocytes that circulate in the peripheral blood until they are taken up by a female anopheline mosquito when it feeds. The gametocytes mature into male and female gametes, fertilization occurs and a motile zygote (ookinete) is formed within the lumen of the mosquito gut, the beginning of a process known as sporogony. The ookinete penetrates the gut wall and becomes a conspicuous oocyst within which another phase of multiplication occurs resulting in the formation of sporozoites that migrate to the salivary glands of a mosquito and are injected when the mosquito feeds on a new host (Figure 4).

This cycle can be 24 hourly (*P. knowlesi*), occurring every two days (tertian fever) in *P. vivax*, *P. falciparum* and *P. ovale* infections, and every three days (quartan fever) for *P. malariae*. This life cycle causes the classic symptom of malaria of paroxysm—a cyclical occurrence of sudden coldness followed by shivering and then fever and sweating. These invaded red cells can be seen in thick and



AFR: WHO African Region; AMR: WHO Region of the Americas; EMR: WHO Eastern Mediterranean Region; SEAR: WHO South-East Asia Region; WHO: World Health Organization; WPR: WHO Western Pacific Region.

Figure 1. *Trends in malaria case incidence rate (cases per 1000 population at risk) globally and by WHO region, 2010–2018. The WHO European Region has reported zero indigenous cases since 2015. Source: WHO estimates.*

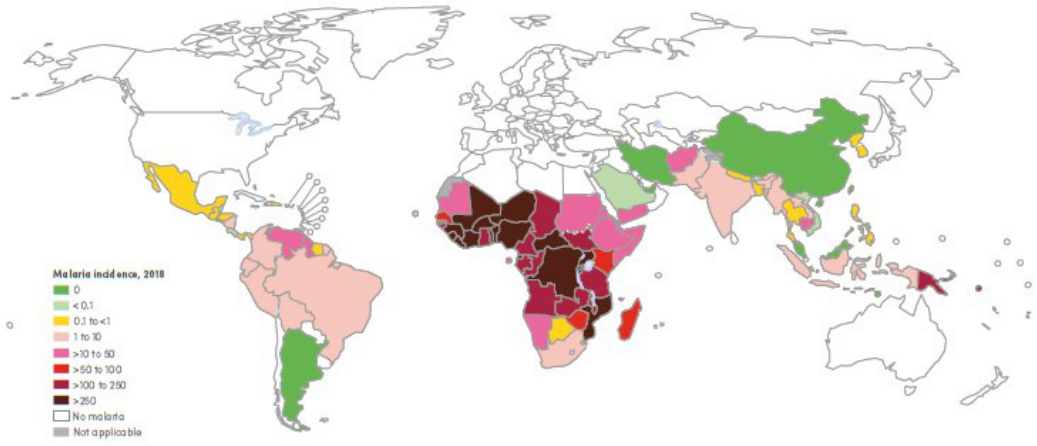


Figure 2. *Map of malaria case incidence rate (cases per 1000 population at risk) by country, 2018 Source: WHO estimates.*

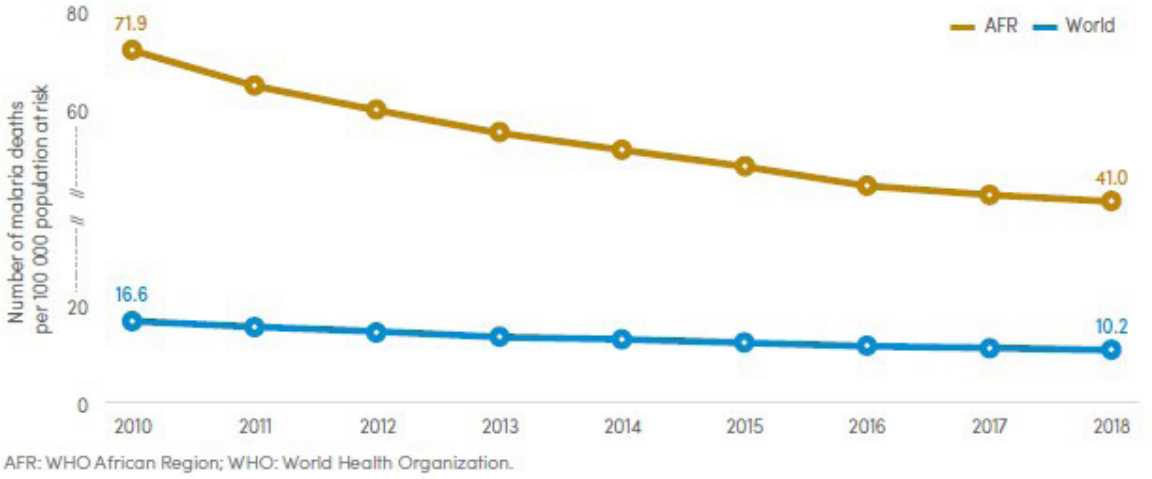


Figure 3. *Trends in malaria mortality rate (deaths per 100 000 population at risk), globally and in the WHO African Region, 2010–2018 Source: WHO estimates.*

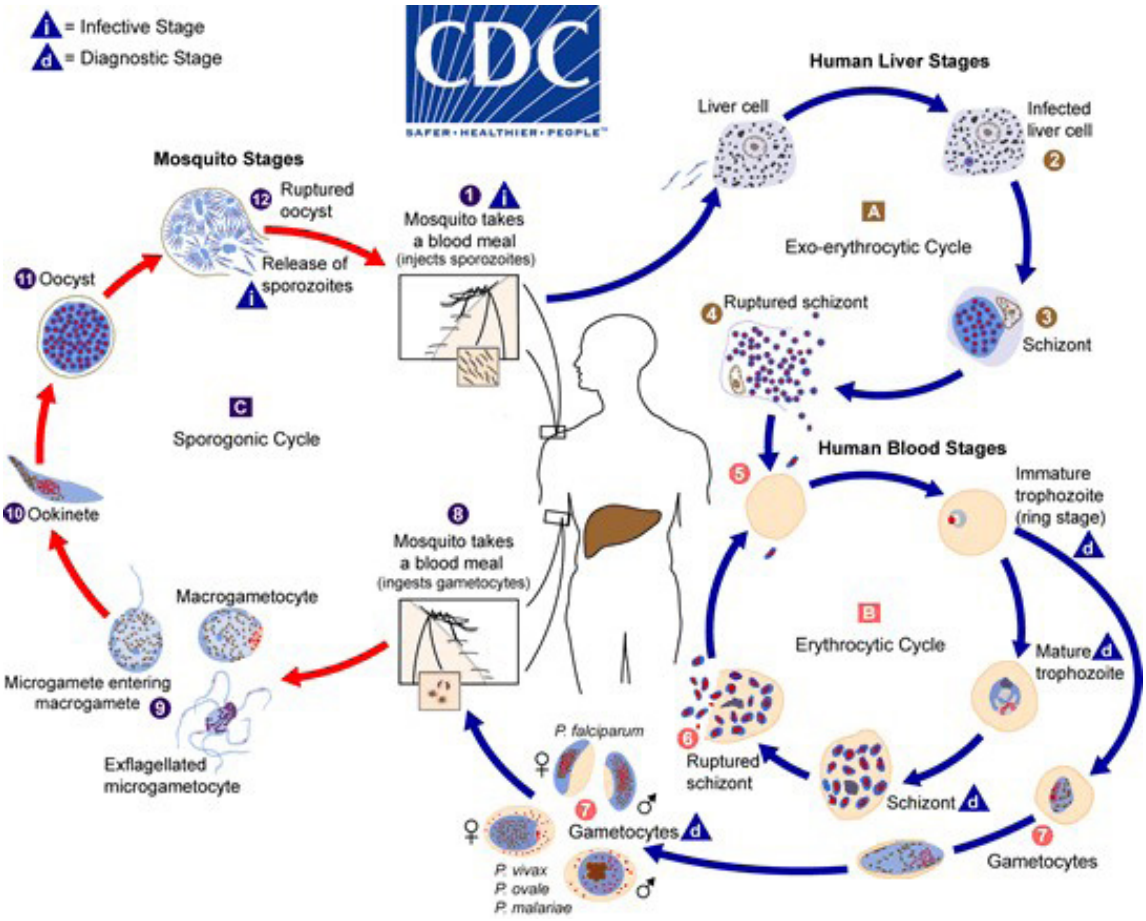


Figure 4. *Life cycle of the malaria parasite.*

Reproduced from CDC https://www.cdc.gov/dpdx/malaria/index.html

thin blood films stained with either Giemsa stain diluted in phosphate buffer at pH 7.2 or Field's stain.

Vaccine development

The development of the stages, penetration of the red cells, schizogony within the host red cells and release of the merozoites have all been extensively studied over the years and have been the targets for vaccine development. Unfortunately, the malaria parasite is much more complex in terms of biology than the viruses and bacteria for which we have vaccines as each of the multiple stages of development in the human host express hundreds of unique antigens.

An immune response targeting one stage may not offer protection against a later stage and different antigens are the targets of protective immunity at different stages.

Furthermore, depending on the life cycle stage and whether the parasite is extra- or intra-cellular, antibody and/or cellular immune responses provide protection.

The targets of the vaccine attempts have been many but the four main approaches to malaria vaccines have been:

- a recombinant protein with adjuvant vaccine aimed at *P. falciparum* pre-erythrocytic stages of the parasite cycle (RTS,S/AS01);
- whole sporozoite vaccines aimed at *P. f* pre-erythrocytic stages (PfSPZ Vaccine and PfSPZ-CVac);
- prime boost vaccines that include recombinant DNA, viruses and bacteria, and protein with adjuvant aimed primarily at *P. f* pre-erythrocytic, but also asexual erythrocytic stages;
- recombinant protein with adjuvant vaccines aimed at *P. f* and *P. vivax* sexual erythrocytic and mosquito stages. (WHO Malaria vaccine rainbow tables).

The malaria vaccine candidate RTS,S/AS01 (Mosquirix) has been tested in a Phase 3 trial with 15,460 children in seven countries in sub-Saharan Africa (Burkina Faso, Gabon, Ghana, Kenya, Malawi, Mozambique, and the United Republic of Tanzania) which began in May 2009 and has now been completed. There were two age categories in the trial: children aged 5-17 months at first dose receiving the RTS,S/AS01 vaccine or a comparator vaccine; and children aged 6-14 weeks at first dose who receive the RTS,S/AS01 vaccine or a comparator vaccine in co-administration with the pentavalent vaccine from the routine immunization schedule.

All children received 3 doses of study vaccines vaccine at one month intervals. The role of a fourth dose 20 months after the first dose was also evaluated (WHO Malaria vaccines).

The European Medicines Agency (EMA) report 2015 showed that:

- Mosquirix provides modest protection against *P. falciparum* malaria in children in the 12 months following vaccination;
- it is effective at preventing a first or only clinical malaria episode in 56% of children aged between 5-17 months and in 31% of children aged 6-12 weeks;
- the efficacy of the vaccine decreased after one year.

Despite its limited efficacy, the benefits of Mosquirix outweigh the risks in both age groups studied and the benefits of vaccination may be particularly important among children in high-transmission areas in which mortality is very high. As Mosquirix does not offer complete protection, and the protection it provides decreases in the longer term, it is important that established protective measures, for example insecticide-treated bed nets, continue to be used in addition to the vaccine (EMA 2015).

WHO, upon review of the data, recommended pilot implementation studies to be conducted for further evaluation of implementability of a four dose schedule in children aged 5-17 months at first dose and further evaluation of the risk/benefit profile.

The future

"*For too long, malaria eradication has been a distant dream, but now we have evidence that malaria can and should be eradicated by 2050*," said Sir Richard Feachem, co-chair of The Lancet Commission on malaria eradication and Director of the Global Health Group at the UCSF. "*This report shows that eradication is possible within a generation. But to achieve this common vision, we simply cannot continue with a business as usual approach. The world is at a tipping point, and we must instead challenge ourselves with ambitious targets and commit to the bold action needed to meet them*" (Feachem *et al* 2019).

Writing in a linked comment, Dr. Tedros Adhanom Ghebreyesus, Director-General of the WHO, says: "*The Lancet Commission makes a bold call for eradicating malaria by 2050. I would be thrilled to see this global scourge eradicated even earlier. But we will not achieve eradication within this time frame with the currently available tools and approaches...The good news is that we,*

the global malaria community, know what we need to do" (Ghebreyesus 2019).

The UCSF Report proposes three ways to eradicate malaria:

- existing ways such as bed nets, insecticides and medicines should be used more smartly;
- new tools such as vaccines should be developed;
- governments in both malaria-affected and malaria-free countries need to boost investment by about $2 billion a year to accelerate progress.

This may occur over the next 30 years but of course other factors that may impede the progress of eradication such as climate change (particularly global warming), malaria mutation and drug resistance and the world economy. Malaria was present in many other countries only a century or so ago – can it return?

Conclusion

Even though our knowledge of malaria and the fight against infection of this parasite has dramatically reduced the incidence and mortality rate over recent years, there is still a long way to go and many influences and factors to overcome. In the meantime, we as scientists, have an important role to play in identifying and speciating the parasites when travellers, refugees and immigrants present at our GPs and hospitals complaining of fevers, chills and sweating episodes.

Our contribution to their prompt treatment will help reduce morbidity and mortality.

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Journal-based CPD No. 68
Page 1 of 1

Questions relating to the article *'Abo blood group secretor status correlates with autoimmune diseases in Myanmar patients'* at page 50 of this issue.

1.	In the ABH blood group system secretors release H, A or B antigens into body secretions the mechanism of which is governed by the fructosyltransferase 3 *(FUT3)* gene.	True/False
2.	The genotype of secretors is Se/Se or Se/se. Secretors secrete the A antigen which is processed into A and/or B antigens depending on their ABO genotype.	True/False
3.	The *FUT2* gene (Se locus) is located on chromosome 19 at 19q13.3.	True/False
4.	The enzyme fucosytransferase catalyzes Type-1 and Type-3 glycan to form the H antigen in body secretions (Stanley and Cummings 2017).	True/False
5.	About 80 % of individuals are secretors and they secrete ABH antigens into their body secretions except for cerebrospinal fluid.	True/False
6.	Dickey and colleagues in 1994 found that the non-secretor state is significantly associated with coeliac disease (Dickey *et al* 1994).	True/False
7.	The suspensions were mixed by tapping the tubes and left undisturbed for 15 min before centrifugation for 1 min at 112 g.	True/False
8.	Anti-A, anti-B (IgM/Swemed Diagnostics) and anti-H (extract from *Ulex europaeus* seed, National Blood Centre, Thai Red Cross Society) were used.	True/False
9.	These carbohydrate structures on cell surfaces can be exploited by pathogenic microorganisms to gain entry to the cell or to facilitate microorganism survival (Daniels 2013a)..	True/False
10.	Anti-H which is specific for fucose and GlcNAc is produced from *Ulex europaeus* (gorse) seed.	True/False

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Page 1 of 1

Questions relating to the article *'The clinical features and dermoscopic patterns"* at page 56 of this issue.

1.	Cutaneous metastases are rare clinical manifestations of malignancy with metastasis.	True/False
2.	Early diagnosis of skin metastases can be a predictor in determining prognosis and in some cases even detecting the presence of a hidden primary malignancy.	True/False
3.	The cutaneous metastases of internal malignancies are uncommon, which varies between 0.7 to 10 % of patients (Lookingbill *et al* 1990).	True/False
4.	The incidence of cutaneous metastases varies in different population, and previous research showed that as many as 29% had cutaneous metastases.	True/False
5.	In women, the most common etiology included breast cancer (89%), colorectal cancer (39%), melanoma (55%) and ovarian carcinoma (4%) (Brownstein and Helwig 1972).	True/False
6.	Skin metastases involve the head and neck (28%), trunk (40%), extremities (18%) and other sites (14%) with an age range of 37-86 years, with an average of 62 years (Sariya *et al* 2007).	True/False
7.	The patient was previously treated with topical antibiotics and intravenous ceftriaxone (2 grams / 24 hours), Heparin (1000 IU/hour).	True/False
8.	Laboratory examination showed anemia 10 mg/dl, hyperglycemia 233 mg/dl, hypoalbuminemia 2.5 g/dl, and leukocytosis 15,410/ul.	True/False
9.	Results suggested glandular epithelium origin which partially showed squamous differentiation.	True/False
10.	Metastasis in nasal area is reported to be an early sign of ovarian carcinoma, but can also appear into the chest, back, vulvovaginal area, arms and both legs.	True/False

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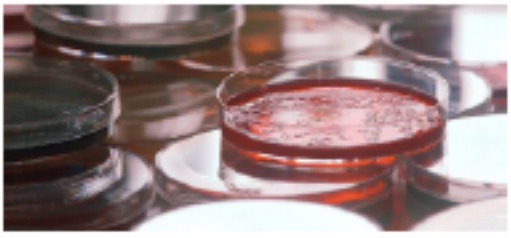

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- Introduction
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Clearly state the purpose of the article leading the reader from the known to the unknown. Summarise the rationale for the study and state the question to be answered as appropriate. Give only strictly pertinent references, and do not review the subject extensively.

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Present the materials and methods in a logical sequence. Describe the selection of the observational or experimental subjects (patients or experimental animals, including controls) clearly. Notification of ethics approval must be given where relevant. Identify the methods, apparatus and procedures in sufficient detail to allow other workers to reproduce the results. Give references to established methods, including statistical methods. Adequately describe new or substantially modified methods. Identify precisely all drugs and chemicals used, including generic name(s), dosage(s), and route(s) of administration. Do not identify patients or hospitals without consent.

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Acknowledgements

Acknowledge individuals who have made substantial contributions to the study including technical work and financial support. Authors are responsible for obtaining consent from all the individuals acknowledged by name as inclusion may be interpreted as an endorsement of the article's contents.

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Use only standard abbreviations (see list of commonly used abbreviations).

Avoid abbreviations in the title. The full term for which an abbreviation stands must precede its first use in the text unless it is a standard abbreviation for a unit of measurement.

Report measurements in the units in which the measurements were made. In most countries the International System of Units (SI) is standard.

Commonly used abbreviations

Abbreviation or Symbol	Standard Units of Measurement
g	gram
g	gravity
Hz	hertz
h	hour
IU	international unit
K	kelvin
kg	kilogram
L	liter, litre
m	meter, metre
min	min
M	molar
mL	millilitre
mol	mole
N	newton
nm	nanometre
p	probability
rpm	revolutions per min
s	second
wk	week
yr	year

Additional information

The following are useful sources of information. The first two publications are used by the AJMS as standard references.

Style Manual Committee. Council of Biology Editors. *Scientific style and format: the CBE manual for authors, editors, and publishers.* 6th ed. Cambridge University Press, 1994.

Style manual for authors, editors and printers. 6th ed. John Wiley & Sons Australia Ltd, 2002.

O'Connor M, Woodford FP. *Writing scientific papers in English: an ELSE-Ciba Foundation guide for authors.* Amsterdam, Oxford, New York: Elsevier-Excerpta Medica, 1975.

Day RA. *How to write and publish a scientific paper.* Philadelphia, Institute for Scientific Information Press, 1979.

Zeiger M. *Essentials of writing biomedical research papers*. 2nd ed. New York, McGraw-Hill, 2000.

Matthews JR, Matthews RW. *Successful scientific writing: a step-by-step guide for the biological and medical sciences*. 3rd ed. Cambridge, Cambridge University Press, 2007 [Also available in eBook format.]

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