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**Title:** Chronic Mucocutaneous Candidosis due to Gain-of-Function Mutation in STAT1

**Running title:** CMC due to Gain-of-Function Mutation in STAT1

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**Abstract:** Chronic mucocutaneous candidiasis (CMC) is a heterogeneous group of primary immunodeficiency diseases characterised by susceptibility to chronic or recurrent superficial *Candida* infection of skin, nails and mucous membranes. Gain-of-function mutations in the STAT1 gene (STAT1-GOF) are the most common genetic aetiology for CMC, and mutation analysis should be considered. These mutations lead to defective responses in type 1 and type 17 helper T cells (Th1 and Th17), which, depending on the mutation, also predispose to infection with Staphylococci, Mycobacteria & Herpesviridae. We describe the clinical and genetic findings for three patients with CMC due to gain-of-function mutations in the STAT1 gene.

## Introduction

Chronic mucocutaneous candidiasis (candidosis) (CMC) is a heterogeneous group of primary immunodeficiency diseases characterised by susceptibility to chronic or recurrent superficial *Candida* infection of skin, nails and mucous membranes (Glocker and Grimbacher, 2010). Mutations in a number of genes, e.g. STAT1, AIRE, CARD-9, Dectin-1, have been associated with susceptibility to CMC (van de Veerdonk, 2011; Ferwerda et al, 2009; Glocker et al, 2009; Chu et al, 2012; Capalbo et al, 2013) with some relationship between genotype and disease phenotype.

Mutations in the gene encoding Signal Transducer and Activator of Transcription-1 (STAT1) protein are the most commonly reported cause of CMC. STAT1 proteins are key components of the adaptive immune response to pathogenic microorganisms. The balance of STAT1 and STAT3 signaling is one factor determining whether activated T-helper cells develop along a Th1 or Th17 pathway and affects the phenotype of the ensuing immune response. Mutations in STAT1 result in either loss-of-function (LOF) or gain-of-function (GOF) (Boisson-Dupuis et al, 2012; Bustamante et al, 2015; van de Veerdonk et al, 2011). STAT1-GOF mutations are associated with a variety of phenotypes including CMC, fungal infections other than candidiasis, bacterial and viral infections, mycobacterial infections, autoimmune disorders, as well as carcinomas and aneurysms (Liu et al, 2011; van de Veerdonk et al, 2011; Toubiana et al, 2016). Heterozygous mutations in coiled-coil domain (CCD) and DNA-binding domain (DBD) of STAT1 are identified in approximately 40% of patients with CMC, of which 62% are confined to the CCD and 35% to the DBD (Toubiana et al, 2016), meaning only 3% lie outside of these domains (Figure 1).

In this series, we describe the genetic and clinical findings of three patients with CMC due to novel mutations in the STAT1 gene, two of which lie outside of the typical regions. All three cases presented to specialists in Oral Medicine, and were subsequently referred to immunology for systematic investigation (Table 1). The local laboratory performed routine tests, including serum immunoglobulins, serum protein electrophoresis, IgG subclasses, lymphocyte subsets and HIV testing. Multiple specialist centres performed lymphocyte proliferation to *Candida* antigens, measurement of Type 1 cytokines and anti-cytokine antibodies, and STAT1 genetic testing.

### **Case 1**

A 34-year-old Caucasian female was referred with a history of recurrent oral and vulvovaginal candidiasis and recurrent dermatophytosis of the hands and feet since childhood. The patient reported oral discomfort, exacerbated by acidic foods. The medical history was otherwise

unremarkable with no history of autoimmunity. The family history was positive for mucocutaneous fungal infection in a number of first-degree relatives. Clinical examination showed erythema of the hard palate and candidal pseudoplaques involving the dorsum tongue (Figure 2A &B). Investigations (Table 1) demonstrated biochemical hypothyroidism, with low T4 and raised TSH, a mild normocytic anaemia and impaired T-cell proliferation to candidal antigens. An oral isolate of *C. albicans* demonstrated resistance to fluconazole, intermediate resistance to itraconazole and susceptibility to other agents tested (echinocandin, flucytosine, voriconazole and amphotericin). Targeted sequencing of the STAT1 gene revealed a heterozygous sequence variant c.209G>C p.(Arg70Pro) in exon 4.

Induction therapy with systemic itraconazole 200mg daily for six months was administered followed by suppressive therapy of itraconazole for one week of every month for a further six months. The oral candidiasis is presently controlled with chlorhexidine mouthwash twice daily and nystatin suspension daily, with itraconazole reserved for breakthrough periods. There was a significant improvement in clinical signs with no further symptoms reported at 18-month follow-up (Figure 2C & D).

## Case 2

A 66-year-old male of Italian descent was referred by Oral and Maxillofacial Surgery with a history of recurrent oral candidiasis. He reported generalised soreness of the oral cavity. The patient had a history of chronic oral candidiasis since the age of five, chronic onychomycosis involving the fingernails and recurrent staphylococcal folliculitis in adulthood. He had no history of deep-seated infections or sinopulmonary infections. The medical history was significant for recurrent folliculitis due to *Staphylococcus aureus*, tongue squamous cell carcinoma (diagnosed at the age of 60), non-insulin dependent diabetes mellitus, gastro-oesophageal reflux disease and Raynaud's phenomenon. There was no immediate family history of recurrent, unusual or recalcitrant infections.

Examination revealed a number of raised erythematous lesions in the beard distribution. His fingernails were brittle with some nailbed lifting. On intraoral examination, an extensive dense white plaque involving the dorsum tongue and erythema of the hard and soft palate were noted (Figure 3A & B).

Investigations (Table 1) revealed; impaired IL-17 production in response to polyclonal T-cell stimulation, autoantibodies to IL-29 and IL-21 and an oral *C. albicans* isolate was susceptible to all azole antifungals tested. Cutaneous infection with *S. aureus* was demonstrated. Gastroscopy revealed no evidence of candidiasis. Targeted sequencing of the STAT1 gene identified a heterozygous sequence variant c.2159C>T p.(Thr720Ile) in exon 24.

The patient underwent induction therapy with itraconazole 200mg daily. He remained asymptomatic with a mild clinical improvement in the appearance of the dorsum tongue and palate (Figure 3C & D). After 5 months of induction therapy, he developed a recurrent squamous cell carcinoma of the tongue (RT4NOMO). The oral candidiasis is currently controlled with topical miconazole and chlorhexidine oral rinse while he receives palliative radiotherapy.

### **Case 3**

A 55-year-old black male of Ugandan descent reported intermittent soreness of the buccal mucosa and tongue to acidic foods. He described a history of recurrent dermatophytosis of the hands for approximately 25 years and recurrent oral candidiasis for 15 years. He had a history of systemic sclerosis associated with pulmonary fibrosis and recurrent oesophageal strictures requiring dilatation, and coeliac disease. He had two daughters, both of whom had recurrent oral candidiasis and onychomycosis. Examination revealed facial telangiectasia, microstomia, cutaneous sclerosis of the neck and abdomen, sclerodactyly and evidence of previous digital ulceration. Oral examination demonstrated bilateral angular cheilitis, diffuse erythema of the mucosa with pseudomembranous candidiasis and superficial ulceration (Figure 4A).

Investigations (Table 1) revealed: an oral *C. albicans* isolate was resistant to fluconazole with intermediate resistance to itraconazole and voriconazole. Targeted sequencing of the STAT1 gene revealed a heterozygous sequence variant c.850G>A p.(Glu284Lys) in exon 10.

The patient underwent induction therapy with voriconazole 200mg twice daily for six months and suppressive therapy with amphotericin lozenges 10mg twice daily, chlorhexidine mouthwash once daily and voriconazole reserved for breakthrough symptoms. He remains asymptomatic with a considerable improvement in clinical appearance (Figure 4B) at 20-month follow-up.

## Discussion

### *The anti-Candida host immune response*

Chronic mucocutaneous candidiasis (CMC) describes a syndrome of chronic or recurrent superficial infection of skin, nails or mucous membranes by *Candida* species, typically *C. albicans*. *Candida* species are commensal yeasts that can be isolated from the oral cavity of up to 80% of healthy individuals (Villar and Dongari-Bagtzoglou, 2008). Immune defects identified in patients with CMC have helped identify the crucial components of the normal mucosal immune response to *Candida*. *C. albicans* is a dimorphic yeast that exists as blastoconidia (yeast cells), as pseudohyphae and as hyphae. Its ability to switch between different forms is essential for this fungus to remain virulent, and is modified by local and systemic environmental factors. An array of innate immune pathogen recognition receptors (PRRs), including Dectin-1, Galectin-3 and the Toll-like receptors TLR2 and TLR4 recognise *Candida* and are expressed by a range of cell types including keratinocytes, macrophages, and dendritic cells (Netea et al, 2015). Candidal recognition leads to activation of Th17 T-cells and innate lymphoid cells (ILCs), which, by releasing IL-17 and IL-22, induce mucosal epithelial cells to produce antimicrobial peptides (AMP), such as b-defensins, which in turn disrupt the fungal cell membrane leading to fungal

cell death (Eyerich et al, 2011; Tomalka et al, 2015). Perturbation of any aspect of this complex network can lead to both overgrowth of *Candida* and/or an over-exuberant immune response to *Candida*, both of which may be damaging to the host. Individuals with impaired mucosal immune responses to *Candida* rarely develop invasive fungal disease because other aspects of the immune response, for example phagocytosis, remain generally intact (Lilic, 2012). The Th17 immune response has only recently been described. It is as a key mediator of protection against extracellular microbes, and sits apart from Th1 and Th2 responses that respond to extracellular and intracellular pathogens respectively (Zelante et al, 2007; Conti et al, 2009).

#### *Genetic susceptibility to Candida infections*

CMC is associated with defects involved in *Candida* recognition (e.g. CARD9 and Dectin1), Th17 differentiation (e.g. STAT1 and STAT3 mutations) and IL-17 signalling (e.g. anti-IL-17 autoantibodies) (Milner and Holland, 2013). CMC is a prominent feature of a number of primary immune deficiencies including the hyper-immunoglobulin E syndromes (HIES) and autoimmune polyendocrine syndrome type I (APS-I). Patients with autosomal dominant HIES, have heterozygous loss-of-function (LOF) mutations in the *STAT3* gene, leading to reduced numbers of Th17 cells and impaired production of IL-17A, IL-17F, and IL-22 (Milner et al, 2008; de Beaucoudrey et al, 2008; Ma et al, 2008; Chandesris et al, 2012). Patients with APS-I have LOF mutations in the *AIRE* gene, which results in failure to delete auto-reactive T-cells, and the development of high titres of autoantibodies against Th17 cytokines including IL-17F (75%) and IL-17A (41%) (Aaltonen et al, 2007; Puel et al, 2010; Kisand et al, 2010; Kisand and Peterson, 2011).

Next-generation sequencing (NGS) has facilitated the discovery of novel disease-causing mutations and the genetic diagnosis of patients with primary immune deficiencies including the role of mutations in STAT1 in patients with CMC (Boisson et al, 2015; Picard and Fischer, 2014; Itan Y and Casanova, 2015). Autosomal dominant GOF mutation in STAT1 are now recognised as the most common cause of CMC and account for more than half of cases (Puel, 2012; Liu L et al, 2011; van de Veerdonk et al, 2011; Toubiana et al, 2016). STAT1-GOF mutations increase STAT1 phosphorylation and cause increased production of the STAT1-dependent cytokines IFN- $\alpha/\beta$ , IFN- $\gamma$ , and IL-27 (Liu et al, 2011; Takezaki et al 2012; Boisson et al, 2015). These cytokines affect gene transcription in naïve T-cells, resulting of maturation of CD4+ T-cells into a Th1 phenotype rather than a Th17 phenotype (Villarino, 2010; Takezaki et al 2012; Boisson et al, 2015). The genetic defect ultimately leads to an exaggerated Th1 cell response and a diminished Th17 response.

A range of STAT1-GOF mutations have been described, but the biological relevance of newly described mutations must be confirmed by undertaking functional immunological analysis (Casanova et al, 2014). The functional impact of different STAT1 variants is typically investigated using flow cytometry to measure induction of STAT1 phosphorylation in peripheral blood cells (PBMCs) following incubation with interferon (IFN)- $\alpha$ , IFN- $\gamma$  and interleukin-27. Measuring the frequency of Th17 cells in peripheral blood can also be performed. At present, these investigations are generally only available in research facilities. The majority of STAT1-GOF mutations described to date occur in either the Coiled Coil Domain (CCD) or the DNA-binding domain (DBD). Mutation location does not appear to correlate with the severity of clinical symptoms (Liu et al, 2011; van de Veerdonk et al, 2011; Smeekens et al, 2011; Hori et al, 2012; Soltesz et al, 2013; Frans et al, 2014; Mekki et al, 2014; Mizoguchi et al, 2014; Yamazaki et al, 2014).

The three mutations described in this case series have, to our knowledge, not been reported in the literature or on any polymorphism databases, and have been classified as variants of uncertain clinical significance (VUS). A mutation at the same site as the patient in Case 1 has been described in a patient with CMC (Toubiana et al, 2016), but with a different amino acid substitution; Arginine to Histidine, as opposed to the Arginine to Proline substitution in our case. The functional impact of the Arginine to Proline mutation has been investigated in-vitro, and has been found to result in a gain of function phenotype (R. Doffinger, personal communication). This mutation does not lie in either the coiled coil or DNA binding domain of the STAT1 transcription factor. The mutation in Case 2 lies outside the coiled coil and DNA binding domain and a recent publication states that this is a gain of function mutation (Toubiana et al, 2016). The case 3 mutation variant is in the coiled-coil domain and has not been reported in the literature or on any polymorphism databases.

#### *STAT1-GOF clinical features*

An international STAT1 GOF study group published clinical, genetic, and laboratory findings of 274 patients (Toubiana et al, 2016). The median age was 22 years (range, 1-71 years). The male to female ratio was 1:1. Ninety-eight percent had CMC, with a median age at onset of 12 months (range, 0-24 years). Many patients also experienced bacterial infections (74%), mainly due to *Staphylococcus aureus* (36%), and viral infections (38%), mainly due to *Herpesviridae* (83%). Some patients also suffered from invasive fungal infections (10%), caused by *Candida* species (29%), and mycobacterial disease (6%). Our patient in Case 2 had a history of recurrent folliculitis due to *Staphylococcus aureus*. Autoimmune manifestations were described in 37% of this series and included endocrinopathies (hypothyroidism, insulin-dependent diabetes mellitus), blood cytopenias and connective tissue disease. In our series, hypothyroidism (Case 1), systemic sclerosis (Case 3) and coeliac disease (Case 3) were noted. Arterial aneurysms,

typically of the cerebra vasculature, were documented in 6% of the Toubiana series with the group recommending systematic radiological screening.

#### *CMC & risk of Cancer*

Patients with CMC have an increased risk of both oral and oesophageal cancer (Bakri et al, 2010; Koo et al, 2016), with patients often presenting at a much younger age than those without CMC. In the Toubiana paper, 6% of CMC patients developed cancer at a median age of 43, compared to an age-adjusted expected rate of 1.1%, with the majority developing squamous cell carcinomas of skin, larynx and gastrointestinal tract. *C. albicans*-mediated enzymatic activity results in the production of nitrosamines, products that are carcinogenic and is considered by some to be a significant risk factor for squamous cell carcinoma (Hsia et al, 1981; Krogh, 1990). Other potential mechanisms of carcinogenesis include chronic inflammation caused by the infection itself. It is also well known that STAT1 is involved in tumour genesis and tumour suppression with reports of reduced levels in transformed cancer cells and poorer outcomes if it is absent (Zhang et al, 2014). Our patient in Case 2 had a history of squamous cell carcinoma involving the tongue and developed a recurrence whilst under surveillance.

#### *Treatment & Surveillance*

The mainstay of treatment for patients with CMC is systemic and topical antifungals. Long-term suppressive therapy is often required to prevent recurrence (Husebye et al, 2009; Kirkpatrick, 2001). Fluconazole is well tolerated, easy to administer, with low toxicity and few side effects. However, its penetration into candidal biofilm is poor, leading to low drug concentrations, with a potential for development of drug-resistant strains (Ramage et al, 2002). *Candida* strains resistant to antifungal therapy remains a concern (Brown et al, 2012; Siikala et al; 2010). In the Toubiana series, resistance to at least one antifungal was observed in 39% of patients treated

with long-term antifungal therapy and in 15% of patients treated intermittently, with azole resistance being most common. Echinocandins are highly effective agents against *Candida*, including biofilms (Ramage et al, 2002) but their availability only as an IV formulation and high cost limit their use for treatment of non-life threatening oral candidiasis.

Topical therapy with polyene antifungal agents (nystatin and amphotericin B) is an attractive option; they are potent, fungicidal, have the broadest range of antifungal activity (Andriole, 1999; Wynn et al, 2003), a low incidence of resistance (Kuhn et al, 2002) and have been shown to be effective in patients with CMC (Rautemaa et al, 2008; Porter et al, 1995). However, the four times a day dosing schedule of both nystatin and amphotericin lozenges may impact on patient compliance (Blomgren et al, 1998; Su et al, 2008). In addition, amphotericin B lozenges are not readily available in all countries. Chlorhexidine is another therapeutic option for topical use owing to its broad-spectrum antibacterial and antifungal activity (Ramage et al, 2011). Even at low concentrations, it can be retained in the oral cavity for up to 12 hours due to its high adsorption capacity (Ellepola and Samaranayake, 2001). It appears to inhibit cell wall synthesis by binding to negatively charged groups, resulting in leakage of intracellular material and cell death (Hiom et al, 2002). It also prevents candidal replication and adhesion of *Candida* to epithelial cells. Significant activity against *C. albicans* has been illustrated *in-vitro* and chlorhexidine has been shown to have superior efficacy against *Candida* biofilms when compared with fluconazole (Ramage et al, 2011; Thurmond et al, 1991).

With no effective vaccine for *C. albicans* and increased antifungal resistance, other therapeutic modalities are required. Oral ruxolitinib, a JAK1/2 kinase family protein tyrosine kinase inhibitor, has shown promise, resulting in complete clinical remission on therapy, but with rapid recurrence of symptoms following cessation (Higgins et al, 2015; Mössner et al, 2016; Weinacht et al, 2017). JAK1/2 inhibition inhibits STAT1 mediated intracellular signalling, allowing restoration of STAT3 signalling and development of naïve T-helper cells into a Th17 phenotype (Weinacht et al, 2017). Granulocyte-colony stimulating factor or granulocyte

monocyte-colony stimulating factor may enhance Th17 cell differentiation and recovery from fungal infections (Dotta et al, 2016). Haematopoietic stem cell transplantation (HSCT) is another treatment option, but due to high rates of treatment related morbidity and mortality is reserved for the most severe CMC cases. Four of six individuals who had HSCT for STAT1-GOF mutation died, due to disseminated CMV infection, hemophagocytic lymphohistiocytosis, and pulmonary complications (Toubiana et al, 2016; Aldave et al, 2013). Other immunotherapies such as recombinant IL-17 may become the treatment of choice in the future.

### **Conclusion & Future directions**

The diagnosis of CMC should be considered in any patient with recurrent or chronic candidiasis or infection recalcitrant to conventional anti-fungals. Prompt referral for immunological investigation allows early initiation of treatment and screening and reduces the significant morbidity and mortality associated with this condition. The mainstays of treatment include suppression of *Candida* infection using a combination of intermittent systemic therapy with long term topical suppression, diagnosis and management of associated autoimmune disease, consideration of screening for cerebral arterial aneurysms, urgent referral for endoscopy for patients with dysphagia and odynophagia and regular surveillance of the oral cavity to detect early dysplastic changes.

The clinical spectra of CMC will expand with new mutations almost certainly to be described moving forwards. An ongoing collaborative approach is needed to address many outstanding issues, including validation of new mutations, the phenotypes they produce and clinical trials of treatment.

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**Table 1: Investigations performed for all cases**

Investigation	Case 1	Case 2	Case 3
HIV	Negative	Negative	Negative
Lymphocyte subsets (CD4+ T-cell, CD8+ T-cell, B-cell & NK-cell)	Normal	Normal	Slightly low CD4+ T-cell count 356x10 <sup>6</sup> /L (normal range 455-1320)
Immunoglobulin levels, IgG subclasses & serum protein electrophoresis	Normal	Normal	Polyclonal elevation in IgG (20.7g/L, range 5.5-16.5) and IgA (6.7g/L, range 0.8-4.0), and low IgM (0.18g/L, range 0.4-2.0)
Serum mannan binding lectin	519 mcg/L (heterozygous range)	>4000 mcg/L (normal)	Not done
Lymphocyte proliferation* to standard antigens (phytohaemagglutinin (PHA), and anti-CD3)	Normal	Normal	Impaired: Control 180->19,046 Case 1252 -> 2100
Lymphocyte proliferation* to candidal antigens	Impaired: Control 922-> 10253 Patient 522-> 2944	Normal: Control 833->23,605 Patient 2229->24,495	Impaired / absent: Control 1440 -> 16,363 Patient 1262 -> 1397
Cytokine production assays (IFN $\gamma$ , IL17, TNF $\alpha$ , IL6, IL10, IL12)	Normal	Low production of IL17 in response to polyclonal T-cell stimulation.	-----
Anticytokine antibodies	None detected	IL29 (moderate titre) IL21 (high titre)	-----
Mutation analysis of STAT1 gene (exons 3-25 by direct	Yes	Yes	Yes

<b>sequencing)</b>			
<b>Mutation site</b>	c.209G>C p.(Arg70Pro) Exon 4	c.2159C>T p.(Thr720Ile) Exon 24	c.850G>A p.(Glu284Lys) Exon 10
<b>Mutation located in DNA binding or CC domain</b>	No	No	CC domain
<b>Mouth swabs</b>	C. albicans	C. albicans	C. albicans
<b>Resistance patterns</b>	R: fluconazole	Fully susceptible	R: fluconazole
<b>R - resistant</b>	I: itraconazole		I: itraconazole, voriconazole
<b>I - intermediate</b>			
<b>Other significant results</b>	Biochemically hypothyroid (low T4, elevated TSH),  Normocytic anaemia (Hb 118, normal MCV and MCH)	MRSA from skin lesions	Transient ANA positive (titre 1:160, speckled staining pattern), ENA negative

\* Results reported as counts per minute (CPM). No absolute reference range, test compares proliferation of lymphocytes from patient and a control individual, at background level & following stimulation.

## Figure Legends

**Figure 1:** Signal transducer and activator of transcription (STAT) proteins contain several functional regions. At the N-terminus, there is a region known as the N-domain. This is followed by a coiled-coil domain, the DNA-binding domain, a linker region, the Src-homology 2 (SH2) domain, tail segment domain (TS) and a carboxy-terminal transactivation domain (TA).

**Figure 2:** Pre-treatment images demonstrating erythematous areas involving the dorsum tongue and generalised erythema of the vault of the hard palate (A & B). Post-treatment images demonstrating almost complete resolution (C & D).

**Figure 3:** Dense adherent plaques involving the dorsum tongue with pseudomembraneous candidiasis and erythema of the hard palate (A & B). Post-treatment improvement in the appearance of the hard palate with a mild improvement in the dorsum tongue (C & D).

**Figure 4:** Pseudomembraneous plaques involving the dorsum tongue (A). Post-treatment complete clinical response (B).



