Molecular genetic investigation, clinical features and response to treatment in 18 patients with Schnitzler’s syndrome

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Dan Lipsker??

Grants:

Please Dorota, could you include the next sentence in the grants paragraph.

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Abstract

**Objective.** To characterise the clinical features and response to interleukin-1 (IL-1) blocking therapy in 18 patients with Schnitzler’s syndrome (SchS) identified at a single UK centre. To search for germline and somatic mutations using the next generation sequencing (NGS) technology and for MYD88 gene mutations, in particular the p.L265P variant.

**Methods.** All subjects underwent detailed investigations. The QualityMetric SF36v2® Health Survey was used to measure functional health and well-being from the patient’s perspective. An amplicon-based deep sequencing (ADS) and NGS gene panel were used to search for germline and somatic mutations in the NLRP3 gene and 166 genes associated with inherited autoinflammatory diseases and vasculitis respectively. MYD88 gene was analysed by Sanger and real-time allele-specific oligonucleotide PCR methods.

**Results.** All patients presented with urticarial rash, fever, arthralgia and bone pain; 47% reported weight loss, 40% fatigue and 21% lymphadenopathies. An IgM kappa paraprotein was detected in 83%, IgM lambda in 11% and IgG kappa in 6%. 94% achieved complete response to anakinra which was sustained during median follow-up of 47 months. Genetic testing revealed one patient had the germline NLRP3 p.V198M mutation; no somatic NLRP3, TNFRSF1A or NOD2 mutations were identified and no universal genetic factors predisposing to SchS were found. A low level c.794T>C substitution in the MYD88 gene was detected in the bone marrow of one patient.

**Discussion.** Despite its rarity SchS is an important diagnosis to consider as treatment with IL-1 blocking agents effectively ameliorate the symptoms, thereby dramatically improving patients’ quality of life and abrogates the risk of developing AA amyloidosis.
Our findings do not support the hypothesis that somatic NLRP3 mosaicism contributes to the pathogenesis of SchS, moreover among the 166 genes tested we have not found a common susceptibility factor for this intriguing disease.

**Introduction**

Schnitzler’s syndrome (SchS) is a very rare, adult-onset, apparently acquired autoinflammatory disease. It was first described in 1972 by the French dermatologist Dr Liliane Schnitzler, with almost 300 cases reported to date. A required hallmark of the disease is presence of a monoclonal protein, which is IgM-kappa class in the vast majority of reported cases (classical type), although monoclonal IgG has been identified in a minority (variant type) {de Koning, 2014 #6502}. The clinical phenotype varies to some extent between patients, but the presence of chronic urticarial-looking rash and a monoclonal IgM or IgG paraprotein are the obligate Strasburg criteria for diagnosis {Lipsker, 2010 #5574}. Other less frequent symptoms constituting minor diagnostic criteria include recurrent fever, bone pain, lymphadenopathy, headache, myalgia, arthralgia, fatigue, weight loss, peripheral neuropathy, neutrophilic dermal infiltrate on skin biopsy, leukocytosis and/or elevated plasma C-reactive protein (CRP). Evolution of the usually quite subtle and asymptomatic underlying clonal disorder to lymphoplasmacytic lymphoma, Waldenström macroglobulinemia (WM), or IgM myeloma has been reported to occur in 12% of patients during median follow-up of 13 years from diagnosis {de Koning, 2014 #6502}.

Clinically, with the exception of symptoms beginning in adult life, SchS bears a striking resemblance to the cryopyrin-associated periodic syndrome (CAPS). This autosomal dominant disorder is caused by gain-of-function mutations in the NLRP3 gene and is characterised by an urticaria-like rash from early infancy, fever and inflammation involving many organ systems, which is associated with a significant risk of AA amyloidosis in the long term. NLRP3 encodes a key component of the NLRP3-inflammasome and CAPS-associated mutations result in
marked upregulation of the inflammasome and substantially increased production of IL-1beta. A further similarity between SchS and CAPS is their dramatic response to IL-1 blocking therapies, implying a pivotal role of excessive IL-1 production in their pathogenesis {Hawkins, 2003 #2823} {Lachmann, 2009 #5480} {Hoffman, 2008 #4248}. A number of IL-1 inhibiting agents are available, but none have yet been specifically licenced for the extremely rare indication of SchS. To date, anakinra (recombinant IL-1 receptor antagonist) has been used most often for the treatment of patients with SchS.

Despite substantial similarities between CAPS and SchS, the precise pathogenetic mechanisms of the latter remains obscure, in particular the interplay between the monoclonal protein and an increased IL-1beta production. Thus far no genetic influence has been identified in SchS, although speculation about the contribution of genetic factors has been fuelled by the finding of the common NLRP3 p.V198M variant in two patients with the classical type SchS {Loock, 2010 #5708; Rowczenio, 2013 #6473}, and the detection of myeloid-restricted somatic NLRP3 mosaicism in two patients with the variant-type SchS {de Koning, 2015 #6599}.

Recently, the p.L265P mutation in the MYD88 gene has been recognised as a hallmark of WM and it is also thought to be a risk factor of the progression from monoclonal gammopathy of undetermined significance (MGUS) to WM. MYD88 is an intracellular adaptor protein that is essential for signalling mediated by the IL-1, IL-18 and toll-like receptors (with exception of TLR3) and the activation of transcription factor NF-kappa B. The MYD88 p.L265P mutation plays a major role in the growth and survival of Waldenström's cells by stimulating uncontrolled activation of a protein complex containing IL-1 receptor-associated kinase 1 (IRAK1) and IRAK4, resulting in increased NF-kappa B signalling {Treon, 2012 #6887} {Varettoni, 2013 #6889}. The rate of progression to haematological malignancy in patients with SchS appears broadly comparable with that of IgM MGUS (18% at 10 years) {Kyle, 2003 #6895} although no one has yet systemically sought MYD88 mutations in SchS.
We report here a study of the clinical features and response to treatment with IL-1 blockade in eighteen patients with SchS identified at a specialist UK centre. The recognition that adult-onset CAPS may be caused by somatic NLRP3 mutations and the close clinical similarity to SchS raises the possibility that somatic NLRP3 mosaicism could contribute to the disease pathogenicity, as de Koning et al previously reported {de Koning, 2015 #6599}. To address this hypothesis, we performed amplicon-based deep sequencing (ADS) in search for somatic NLRP3 mutations in our cohort of patients. Moreover, we have looked for a common susceptibility factor by targeting 166 genes associated with inherited autoinflammatory diseases and vasculitis. Given its involvement in the IL-1 pathway we also explored the possibility that subjects with SchS might possess variants in the MYD88 gene, in particular the previously reported p.L265P mutation.

Patients and Methods
Between 2000 and 2015, 18 patients referred to the autoinflammatory disorders clinic at the National Amyloidosis Centre (NAC) have been diagnosed with SchS according to the Strasbourg criteria. These patients underwent comprehensive clinical and laboratory investigations including routine blood and urine biochemistry, a search for a monoclonal protein by immunofixation electrophoresis of serum and urine, and serum free light chains, serial measurements of inflammatory markers serum amyloid A protein (SAA) and C-reactive protein (CRP) and skin and bone marrow biopsies.

Genetic studies
All genetic analysis were performed on DNA samples isolated from whole blood. The NLRP3 gene was analysed by Sanger sequencing and ADS in all patients; for Sanger sequencing exons 3, 4 and 6 of NLRP3 gene were amplified by PCR and sequenced with the
Big Dye Terminator v 3.1 Read Reaction Cycle Sequencing kit as previously described {Rowczenio, 2013 #6473}; for ADS the amplicons of each DNA sample were obtained using a conventional PCR amplification, with the appropriate tags to identify each sample and sequenced on an IonTorrent platform.

Targeted gene panel sequencing

Additionally patients with SchS underwent Sanger sequencing of the exon 5 of MYD88 gene (NCBI accession NM_002468.4) on DNA extracted from whole blood and in two cases we were able perform these studies on both the blood the bone marrow samples using reagents and methods outlined elsewhere {Varettoni, 2013 #6889}. The MYD88 gene was analysed on the bone marrow samples from two cases by real-time allele-specific oligonucleotide PCR (ASO-RQ-PCR) with the use of primers and a probe published previously (Ref). In brief, two PCR reactions were set up for each sample: one for the detection of the mutant allele using the common forward primer and a probe, and the reverse primer was designed for the detection of p.Leu265Pro mutation. The second PCR amplified the wild type allele as a control of the DNA quality using the wild-type reverse primer and common forward primer and a probe. Each reaction was carried out in a final volume of 25 ul, containing 300nM of each primer (forward and reverse wild-type or mutated), 200nM of the probe, 1xQuantitect Master Mix (Qiagen), 0.4% Bovine Serum Albumin and 20ng of genomic DNA. The PCR reactions were performed in a 7500 Fast Real-Time PCR System (Applied Biosystems) and consisted of an initial denaturation step of 10 minutes at 95°C, followed by 50 cycles of 95°C for 15 seconds and 62°C for 60 seconds using the standard programme. All samples were tested in triplicate and three wells containing reaction mix and water only (no template) were used as a control to detect contamination. Dilutions (1/10, 1/50, 1/100 and 1/500) of a positive
control DNA sample known to harbour the p.Leu265Pro mutation at approximately 40% were analysed along with normal control and patient samples. Data was analysed using the standard 7500 analysis software.

**Quality of life (QoL) assessments**

The QualityMetric SF36v2® Health Survey is designed to measure functional health and well-being from the patient’s perspective. There are eight health domains (physical functioning, role physical, bodily pain, general health, social function, role emotional, mental health and vitality) which are scored individually out of 100 points, and the result expressed in comparison to American norms. Scores closer to 100 represent a better QoL and a change of 10 points or more in a domain between administrations is considered clinically significant. Patients were asked to complete the surveys before starting treatment and at various time-points on treatment.

**Results**

**Patients**

Clinical and laboratory findings in our cohort are enclosed in table 1. The median age at disease onset was 51.3 years (range 37-79). All patients were of British Caucasian ancestry and two thirds were male. All presented with urticarial skin lesions, constitutional upset fever accompanied by arthralgia and bone pain. Additional symptoms included weight loss (47%), fatigue (40%), and lymphadenopathy (21%). Median baseline CRP level in our cohort was 55 mg/l (range 18-257). Sixteen patients had skin biopsies, which were reviewed by an international expert Dan Lipsker, and all were consistent with SchS.

One patient was diagnosed with AA amyloidosis and died of complications of renal failure before treatment with anakinra. The median time from onset of symptoms to initiation of treatment with anakinra in the remaining 17 cases was 62 months (range 21-276); 94% reported
disappearance of all symptoms accompanied by normalisation of their plasma CRP concentration to below 10 mg/l. Responses have been maintained for a median treatment duration of 47 months (range 5-106). No patients have discontinued treatment and adverse events have been confined to minor infections that cannot be directly attributed to anakinra therapy. Results of the Health Survey Questionnaire in our cohort prior to starting anakinra and following 3 – 4 months treatment are shown in Figure 1. A low level paraprotein was identified in each case, of IgM kappa type was in 83%, IgM lambda in 11% and IgG kappa in 6%. To date, clonal disease progression requiring chemotherapy has not occurred in any patient, and doubling of the paraprotein concentration has occurred in only one case.

**Genetic studies**

Sanger sequencing and ADS of the NLRP3 gene confirmed our previously reported patient with the heterozygous, germline p.V198M variant {Rowczenio, 2013 #6473}, but no additional nucleotide alternations, including somatic NLRP3 variants, have been identified. This result was verified by analysing the 166 gene panel, which also demonstrated absence of somatic mutations in the TNFRSF1A or NOD2 genes and did not identify a universal genetic factor predisposing to SchS. Table 1 includes variants identified in our cohort with minor allele frequency <0.1% (allele frequency was obtained from 1000 genome project and Exome Aggregation Consortium (ExAC) public databases). These are known pathogenic mutations or variants of unknown significance identified in the following genes: NOD2 (Nucleotide-binding oligomerization domain 2), CARD14 (caspase recruitment domain family, member 14), IL10 (Interleukin 10), PLCG2 (Phospholipase C, Gamma-2), TRAP1 (Tumour necrosis factor receptor-associated protein 1), NOTCH1 (Neutrogenic locus notch homolog protein 3), TGFBR1 (Transforming growth factor-beta receptor, type 1), TGFBR2 (Transforming growth factor-beta receptor, type 2), NLRP7 (NLR family, pyrin domain containing 7), COL7A1
(Collagen VII, Alpha-1 Polypeptide), COL5A1 (collagen, type V, alpha 1), COL4A1 (Alpha-1 chain of type IV collagen), COL3A1 (Collagen, type III, alpha 1), LYST (lysosomal trafficking regulator), RNF213 (ring finger protein 213), FBN1 (Fibrillin1), FBN2 (Fibrillin2) and ITGB2 (Beta-2 integrin chain).

No germline mutation was found in exon 5 of the MYD88 gene by Sanger sequencing in DNA extracted from whole blood samples, though using this method we cannot completely rule out the presence of somatic MYD88 mutations. We had carried out genetic analysis on the two bone marrow samples by ASO-RQ-PCR, which in one patient demonstrated presence of a low level c.794T>C substitution resulting in p.L265P missense mutation.

Discussion

SchS is an extremely rare clinical presentation of a monoclonal gammopathy, usually but not exclusively of IgM isotype. Whilst monoclonal gammopathy is regarded as central to the diagnosis and pathogenesis of SchS, the nature of the association remains unclear. Recently, a single study reported somatic NLRP3 mosaicism in two cases with variant type SchS in whom the mutation was restricted to cells of myeloid lineage {de Koning, 2015 #6599} suggesting that a population of myeloid cells with an acquired NLRP3 mutation would be expected to produce abnormally high quantities of IL-1beta, inducing chronic stimulation and clonal expansion of local B cells expressing IgM or, less commonly, IgG {Dingli, 2015 #6893}. Our current study however, does not support this hypothesis. We have not identified either germline or somatic NLRP3 mutations in any of the 18 SchS patients described here, other than the presence of p.V198M in the NLRP3 gene, a common variant of uncertain significance, in one case. Recently, Zhou et al. and Mensa-Vilaro et al. reported two unrelated adult patients with late onset but otherwise typical CAPS caused by myeloid-restricted somatic NLRP3 mutations, with no monoclonal gammopathy {Zhou, 2015 #6897}{Mensa-Vilaro, 2016 #6885}. 
Similarly, at the NAC, we have identified eight such patients with mosaic \textit{NLRP3} mutations (data not published). There are striking similarities between the two reported SchS cases and the patients with late onset CAPS caused by somatic \textit{NLRP3} mutations, although neither IgM nor IgG paraprotein were found in the latter group. Furthermore, our experience with 42 adult patients (≥ 40 years old) with genetically confirmed CAPS and long standing IL-1-mediated inflammation is that none have developed a MGUS of any isotype. These data are also supported by a similar lack of paraprotein development in 54 adult patients presenting to our centre with untreated TRAPS or FMF. Relevantly, the two reported variant-type SchS patients with somatic \textit{NLRP3} mosaicism had a very low concentrations of IgG paraproteinemia, which was no longer detectable at follow up. Transient low level paraproteinaemia is a well-recognised phenomenon in chronic inflammatory states, but the persistence of IgM paraproteins in SchS and occasional progression to WM or LPL suggests a very different pathogenesis. We suggest that the two reported cases could reasonably be diagnosed with late onset CAPS due to somatic mosaicism rather than variant SchS. Thus, in patients thought to have SchS who do not completely fulfil the Strasbourg criteria we urge a search for mosaic \textit{NLRP3} mutations given the possibility that these patients may indeed have late onset CAPS. Such a diagnosis may facilitate access to treatment since the anti-IL-1 agents anakinra, canakinumab and rilonacept are all licenced for CAPS.

Sequencing of 166 genes associated with inherited autoinflammatory diseases and vasculitis did not reveal a common genetic predisposition for this rare disease, nonetheless several novel and/or pathogenic variants were discovered, although their relevance in SchS needs to be further investigated.

ASO-RQ-PCR of the bone marrow samples collected from two SchS patients, demonstrated that in one we were able to detect a low level c.794T>C substitution resulting in p.L265P missense mutation in exon 5 of the \textit{MYD88} gene. The patient had IgM paraprotein of 16g/L at
baseline, which was the highest in the cohort although a baseline bone marrow was morphologically normal and there has been no increase in the paraprotein during follow up of six months. This result may suggest that the L265P mutation in SchS patients is required for the expansion of the B cell clone, although further study is needed in support for this hypothesis and we would plan to perform the bone marrow analysis on all new SchS cases.

Despite its rarity SchS is an important diagnosis to consider as treatment with IL-1 blocking agents effectively ameliorate the symptoms and abolishes the biochemical inflammatory response and anemia of chronic disease, thereby dramatically improving patients’ quality of life (Fig 1). The treatment is well tolerated and abrogates the risk of developing AA amyloidosis, which is a known complication of any inflammatory disorder associated with sustained overproduction of serum amyloid A protein. To date, AA amyloidosis has been reported in six of the 281 patients with SchS (2%). In our cohort one patient died of AA amyloidosis before treatment with anakinra could be administered. As yet there is no evidence that treatment with any specific blockade of IL-1 adversely affects the behavior of the associated B cell clone. Indeed, there is a report suggesting the opposite, the monoclonal protein concentration having fallen in one SchS patient following anakinra therapy {Kluger, 2008 #6896}, and benefits of anakinra have also been reported in patients with smouldering myeloma evidenced by reduction of the proliferative index {Lust, 2009 #4974}.

To date, clinically significant progression of the clonal B cell disorder has not occurred in any of our patients, and only one has seen a doubling of their low level paraprotein; none have required chemotherapy. Follow up is currently too short to draw any firm conclusions, as yet, as to whether anakinra treatment might beneficially affect clonal proliferation.

Conclusions
Our findings in 18 patients investigated in a single specialist UK centre do not support the hypothesis that somatic NLRP3 mosaicism contributes to the pathogenesis of SchS, moreover among the 166 genes tested we have not found a common susceptibility factor for this intriguing disorder.

Reference:

Figure Legend:

Figure 1. Quality of life before and during treatment with anakinra. Patients were surveyed before starting anakinra, and whilst on treatment for 3 – 4 months. A comparison of the mean scores in each domain before and on-treatment was statistically significant (Mann Whitney p=0.0003) and clinically meaningful improvement, to well above that of healthy age matched USA controls (normalised to 50), were seen in all domains (a change of 10 points or more is considered clinically significant). PF=physical function, RP=role physical, BP=bodily pain, GH=general health, VT=vitality, SF=social function, RE=role emotional, MH=mental health.
<table>
<thead>
<tr>
<th>Patient number</th>
<th>Sex</th>
<th>Age at symptom onset (years)</th>
<th>Rash</th>
<th>Constitutional symptoms (fatigue/fever)</th>
<th>Bone pain</th>
<th>Lymphopenia</th>
<th>CRP mg/dl</th>
<th>IgM</th>
<th>M-protein</th>
<th>k/λ ratio</th>
<th>Bone Marrow histology</th>
<th>Response to anakinra</th>
<th>Duration of anakinra to clonal progression</th>
<th>Clonal progression requiring</th>
<th>Genetics</th>
<th>Gene</th>
<th>Previously reported on Infever database</th>
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<td>1</td>
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<td>36.8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>40</td>
<td>130</td>
<td>3g IgMκ</td>
<td>1.3</td>
<td>No overt LPL</td>
<td>Partial</td>
<td>86</td>
<td>0</td>
<td>NLRP7</td>
<td>p.R156Q</td>
<td>NOTCH1  p.R1279H1; p.S407L2; FBN1  p.V2234M2</td>
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<td>F</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>257</td>
<td>100</td>
<td>3g IgMκ</td>
<td>0.9</td>
<td>No overt LPL</td>
<td>Complete</td>
<td>72</td>
<td>0</td>
<td>COL7A1</td>
<td>p.G636V</td>
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</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>8</td>
<td>135</td>
<td>12g IgMκ</td>
<td>4.1</td>
<td>No overt LPL</td>
<td>Complete</td>
<td>47</td>
<td>0</td>
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<td>p.L801F</td>
<td>RNF213  p.S5122Y2; FBN2  p.I2394T2</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>60</td>
<td>154</td>
<td>7g IgGκ</td>
<td>0.8</td>
<td>15% PC</td>
<td>Complete</td>
<td>28</td>
<td>0</td>
<td>CARD14</td>
<td>p.R682W7/p.E422K2; TRAP1  p.Y444N2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>F</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>120</td>
<td>139</td>
<td>1g IgMκ</td>
<td>1.1</td>
<td>No overt LPL</td>
<td>Complete</td>
<td>45</td>
<td>0</td>
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<td>p.R684W</td>
<td>NLRP7  p.E507V1</td>
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<td>6</td>
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<td>49.6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>89</td>
<td>133</td>
<td>4g IgMκ</td>
<td>1.4</td>
<td>Not done</td>
<td>Complete</td>
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<td>0</td>
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<td>+</td>
<td>0</td>
<td>16</td>
<td>128</td>
<td>ND</td>
<td>2.5</td>
<td>No overt LPL</td>
<td>Complete</td>
<td>82</td>
<td>0</td>
<td>IL10</td>
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<td>0</td>
<td>45</td>
<td>143</td>
<td>1gMα on IF</td>
<td>0.9</td>
<td>No overt LPL</td>
<td>Complete</td>
<td>106</td>
<td>0</td>
<td>FBN2</td>
<td>p.I2394T2; NOTCH1  p.P1377S2; TGFB1  p.1720del2; TGFB2  E150S2; LYST  p.N2971K2</td>
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</tr>
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<td>0</td>
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<td>LPL</td>
<td>Complete</td>
<td>83</td>
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<td>0</td>
<td>49</td>
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<td>3g IgMκ</td>
<td>2.2</td>
<td>LPL</td>
<td>Complete</td>
<td>31</td>
<td>0</td>
<td>COL7A1  p.A1137V; NOD2  p.T190M</td>
<td></td>
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<td>0</td>
<td>79</td>
<td>105</td>
<td>7g IgMα</td>
<td>0.6</td>
<td>No overt LPL</td>
<td>Died of AA amyloid</td>
<td>ND</td>
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<td>+</td>
<td>+</td>
<td>0</td>
<td>40</td>
<td>138</td>
<td>5g IgMκ</td>
<td>8.0</td>
<td>No overt LPL</td>
<td>Complete</td>
<td>50</td>
<td>0</td>
<td>NLRP7</td>
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<td>+</td>
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<td>0</td>
<td>112</td>
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<td>9g IgMκ</td>
<td>15.7</td>
<td>Low grade marginal</td>
<td>Complete</td>
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<td>140</td>
<td>100</td>
<td>8g IgMκ</td>
<td>9.5</td>
<td>No overt LPL</td>
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<td>73</td>
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<td>p.M4633/p.A1006S2; COL7A1  p.R1202H2/p.P1521S2; TGFB2  E150S2; NOTCH3  p.H1133Q2</td>
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<td>+</td>
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<td>Not done</td>
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<td>F</td>
<td>39.7</td>
<td>+</td>
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<td>121</td>
<td>16g IgMκ</td>
<td>1.9</td>
<td>No overt LPL</td>
<td>Complete</td>
<td>7</td>
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<td>MYD88</td>
<td>p.L265F1; NOTCH3  p.P496L2; PLCG2  p.M28L2</td>
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<tr>
<td>17</td>
<td>M</td>
<td>40.7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>17</td>
<td>131</td>
<td>4g IgMκ</td>
<td>5.1</td>
<td>No overt LPL</td>
<td>Complete</td>
<td>5</td>
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<td>COL7A1  p.V1210L2; FBN2  p.T1416A2; TGFB2  E150S2</td>
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<td>18</td>
<td>M</td>
<td>61.2</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>95</td>
<td>8g IgMκ</td>
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<td>No overt LPL</td>
<td>Complete</td>
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<td>COL7A1  p.Y985H1; NOTCH1  p.R1279H1</td>
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1- Somatic mutation; 2- Germline variants of unknown significance; 3- Germline variants previously reported on Infever database
Figure and legend:

Figure 1.

Figure 2.