A family with A20 haploinsufficiency presenting with novel clinical manifestations and challenges for treatment

Timo Hautala^{1,15}, Paula Vähäsalo², Outi Kuismin³, Salla Keskitalo⁴, Kristiina Rajamäki⁵, Antti Väänänen⁶, Marja Simojoki⁷, Marjaana Säily¹, Ilpo Pelkonen⁸, Heikki Tokola⁹, Markus Mäkinen⁹, Riitta Kaarteenaho¹⁰, Airi Jartti¹¹, Nina Hautala¹², Saara Kantola¹³, Päivi Jackson¹⁴, Virpi Glumoff¹⁵, Janna Saarela¹⁶, Markku Varjosalo^{4,17}, Kari K Eklund¹⁸, Mikko RJ Seppänen^{5,19}

¹ Department of Internal Medicine, Oulu University Hospital, Oulu, Finland.

² Department of Pediatrics, PEDEGO Research Unit, Medical Research Center, Oulu University Hospital and University of Oulu, Oulu, Finland.

³ Department of Clinical Genetics, PEDEGO Research Unit, Medical Research Center, Oulu University Hospital and University of Oulu, Oulu, Finland.

⁴ Institute of Biotechnology, Helsinki Institute of Life Science (HiLIFE), University of Helsinki, Helsinki, Finland.

⁵ Clinicum, Faculty of Medicine, University of Helsinki, Helsinki, Finland

⁶ Department of Infection Control, Lapland Central Hospital, Rovaniemi, Finland.

⁷ Department of Obstetrics and Gynecology, Oulu University Hospital, Oulu, Finland

⁸ Hematology Laboratory, Nordlab Oulu, Oulu University Hospital, Oulu, Finland

⁹ Department of Pathology, Cancer Research and Translational Medicine Research Unit, University of Oulu and Oulu University Hospital, Oulu, Finland.

¹⁰ Respiratory Medicine, Research Unit of Internal Medicine, University of Oulu and Medical Research Center Oulu, Oulu University Hospital, Oulu, Finland.

¹¹ Department of Radiology, Oulu University Hospital, Oulu, Finland

¹² Department of Ophthalmology, PEDEGO Research Unit, Medical Research Center, Oulu University Hospital and University of Oulu, Oulu, Finland.

¹³ Department of Dentistry, Oulu University Hospital, Oulu, Finland

¹⁴ Department of Dermatology, Oulu University Hospital, Oulu, Finland

¹⁵ Research Unit of Biomedicine, University of Oulu, Oulu, Finland.

¹⁶ Institute for Molecular Medicine Finland, Helsinki Institute of Life Science (HiLIFE), University of Helsinki, Helsinki, Finland.

¹⁷ Proteomics Unit, Institute of Biotechnology, University of Helsinki, Helsinki, Finland.

¹⁸ Department of Rheumatology, Inflammation Center, University of Helsinki and Helsinki University Hospital. Research Institute, Invalid Foundation. Orton Orthopedic Hospital, Helsinki, Finland.

¹⁹ Department of Clinical Microbiology, Oulu University Hospital, Oulu, Finland.

¹⁹ Rare Diseases Center and Pediatric Research Center, Children and Adolescents and Adult Immunodeficiency Unit, Inflammation Center, University of Helsinki and Helsinki University Hospital, Helsinki, Finland.

Keywords: immunodeficiency, autoimmunity, autoinflammation, lung disease, anemia, anakinra, mycophenolate

Running title: Multiple immunological defects due to HA20

Corresponding author:

Timo Hautala, MD, PhD
Research Unit of Biomedicine
University of Oulu
P.O. Box 20
FIN-90029 OYS, Finland
Phone: +358 40 709 6859
E-mail: timo.hautala@fimnet.fi

57 ABSTRACT

58

59 **Background.** Tumor necrosis factor alpha-induced protein 3 gene (*TNFAIP3*, also called A20)
60 haploinsufficiency (HA20) leads to autoinflammation and autoimmunity. We have recently shown
61 that a p.(Lys91*) mutation in A20 disrupts NF- κ B signaling, impairs protein-protein interactions of
62 A20, and leads to inflammasome activation.

63 **Methods.** We now describe the clinical presentations and drug responses in a family with HA20
64 p.(Lys91*) mutation, consistent with our previously reported diverse immunological and functional
65 findings.

66 **Results.** We report for the first time that inflammasome-mediated autoinflammatory lung reaction
67 caused by HA20 can be treated with IL-1 antagonist anakinra. We also describe severe anemia related
68 to HA20 successfully treated with mycophenolate. In addition, HA20 p.(Lys91*) was found to
69 associate with autoimmune thyroid disease, juvenile idiopathic arthritis, psoriasis, liver disease and
70 immunodeficiency presenting with specific antibody deficiency and genital papillomatosis.

71 **Conclusions.** We conclude that HA20 may lead to combination of inflammation, immunodeficiency
72 and autoimmunity. The condition may present with variable and unpredictable symptoms with
73 atypical treatment responses.

74

75

76 INTRODUCTION

77

78 Haploinsufficiency of A20 (HA20) is an autoinflammatory and autoimmune condition caused by loss
79 of function mutations in tumor necrosis factor alpha-induced protein 3 gene (*TNFAIP3*, also called
80 A20) [1, 2, 3, 4, 5, 6]. Most typically, HA20 patients are reported to suffer from juvenile arthritis or
81 Behçet's like disease. In addition, gastrointestinal complaints, skin involvement, recurrent fever,

vasculitis and uveitis, for example, may associate with HA20. In addition to these clinical consequences, A20 has been shown to regulate T cell maturation [7] and to participate for example in development of steatohepatitis in experimental models [8].

We recently described molecular mechanisms of HA20 caused by a truncating mutation p.(Lys91*). Various biological consequences included reduced NF- κ B signaling, severely impaired protein-protein interactions of A20, and caspase-8-dependent inflammasome activation [9]. The patients had increased secretion of interleukin (IL)-1 β , IL-18 and tumor necrosis factor alpha (TNF- α) after stimulation of peripheral blood immune cells. We now report in detail the clinical presentations in the family and the challenges faced in the treatment of these patients. Clinical details and explicit experience on drug responses in HA20 patients are only briefly described in the literature. We now describe, for example, successful anakinra effect on lung autoinflammation and a mycophenolate mofetil response on HA20 related anemia for the first time.

PATIENTS AND METHODS

A family with HA20 with (Ensembl ENSG00000118503:ENST00000433680:exon2:c.A271T:p.(Lys91*)) was analyzed for their immunological properties, clinical symptoms and responses to disease-modifying antirheumatic drug (DMARD) treatments. The mutation was first found in patient II:1 as described previously [9]. Disease course, treatment regimens, and disease- and treatment-related complications were recorded. Patient consent, or a consent from a parent was obtained by the responsible physician.

Immunological properties including lymphocyte subpopulations and their maturation were analyzed according to accredited protocols (www.nordlab.fi, www.huslab.fi). Humoral immune response was tested with pneumococcus polysaccharide (Pneumovax®) vaccination. T helper 17 (Th17),

interleukin 17 (IL-17), and signal transducer and activator of transcription 3 (STAT3) were analyzed as described [10] .

RESULTS

A family with A20 p.(Lys91*) mutation (Figure 1) was analyzed for their clinical immunology, symptoms and responses to disease-modifying antirheumatic drug (DMARD) treatments. Clinical histories are explained in the Supplement. The symptoms and the responses to various treatments are summarized in Table 1.

Immunological parameters

Blood lymphocyte and B cell counts were within normal range in all family members. Patients II:1 and II:4 had an elevated percentage of activated CD21^{low}CD38^{low} B-cells 13.7 – 15.7% (reference 0.8 – 7.7%) and low percentage of switched memory B cells 3.2 – 6.1% (reference 6.5 – 29.2%). Despite normal plasma IgG of 10.8 – 12.9 g/L (reference 6.7 – 15.0 g/L) levels, they (patient II:1, response to 2/10 serotypes) and (patient II:4, response to 1/10 serotypes) displayed specific antibody deficiency with marginal response to pneumococcal polysaccharide vaccine (Pneumovax®). Immunological parameters including a slightly low IgG2 serum concentration in patient II:1 are summarized in Supplemental Table 1.

Although blood T cell counts were normal, numbers of CD3⁺CD4⁺ T cells ($314 \times 10^6/L$; reference 404 – 1612) and natural killer cells ($30 - 68 \times 10^6/L$, reference 84 – 724) were low in patient II:1. Interleukin-17 expression on anti-CD3/CD28-stimulated CD3⁺CD4⁺ T cells was normal (patient 0.63%, control 0.86%) while the relative numbers of T helper 17 (Th17) CD4⁺CD45RA⁻CXCR3⁻

131 CCR6⁺ memory cells were somewhat low (11.4%; reference 19 – 34%). Signal transducer and
132 activator of transcription 3 (STAT3) phosphorylation was normal [11].

133

134 **Thyroid disease**

135 All affected family members suffered from early-onset autoimmune thyroid disease (AITD)
136 associated with growth delay and positive anti-thyroid peroxidase antibodies. Growth delays were
137 resolved in early childhood after introduction of thyroid replacement therapy.

138

139 **Pulmonary inflammation**

140 At age of 28 years, patient II:1 developed prolonged fever and respiratory distress. No evidence of
141 infection nor responses to broad-spectrum antibiotics were seen. High-resolution computed
142 tomography of her chest was consistent with follicular bronchiolitis or lymphatic hyperplasia (Figure
143 2 A-B). Anakinra treatment (100 mg x 1) led to a rapid and complete resolution of the pulmonary
144 nodules and respiratory symptoms (Figure 2 C-D).

145

146 **Anemia**

147 At age 30, patient II:1 developed acute anemia (hemoglobin 72 g/l, mean cellular volume (MCV) 85
148 fl) followed by peripheral blood reticulocytosis and macrocytosis (MCV 100 – 102 fl). The
149 parameters associated with anemia are described in detail in Supplement. Responses to blood
150 transfusions were at best modest (Supplement Table), there was no active bleeding, and the condition
151 was inconsistent with known inflammatory or other causes for anemia. Serum hepsidin levels were
152 low, plasma erythropoietin was stimulated, and she exhibited an active erythropoiesis in her bone
153 marrow. Markers for hemolysis (Coombs test, serum haptoglobin, plasma hemoglobin, urine
154 hemosiderin) were negative and criteria for hemophagocytic lymphohistiocytosis were not met. There
155 was no response to intravenous immunoglobulin (IgG) treatment or to IL-1 (canakinumab), anti TNF-

156 α (etanercept) or JAK inhibition (tofacitinib) [12]. Although her anemia was controlled with ≥ 20 mg
157 of daily oral prednisolone, she was unable to tolerate prolonged treatment. After numerous treatment
158 attempts, mycophenolate mofetil (500 mg two times per day) seemed to stabilize her blood
159 hemoglobin concentration (Supplement Table).

160

161 **Liver involvement**

162 Patient I:1 had developed liver disease of uncertain etiology. She had been advised to avoid alcohol
163 and medications with potential of liver toxicity. At age 46, she had experienced progression of the
164 liver disease and she had been diagnosed with cirrhosis of the liver, eventually causing her death.
165 Patient II:1 underwent abdominal magnetic resonance imaging (MRI) in which iron accumulation
166 into liver was suggested. Liver biopsy confirmed hepatosteatorosis and an iron load in Kupffer cells
167 and sinusoidal macrophages (Figure 2 E-F). Mild elevations in plasma alanine aminotransferase (36
168 – 68, reference <35 units/liter) and plasma bilirubin concentrations (29 – 44, reference <25 $\mu\text{mol/l}$)
169 were observed. No sign of liver dysfunction or fibrosis was found.

170

171 **Oral and genital ulceration**

172 The patient II:1 had suffered from aphthous stomatitis and genital ulcers for several years. Oral
173 aphthous disease responded very favorably to TNF- α inhibitor etanercept although this treatment was
174 ineffective against anemia. She developed chronic and resistant genital papillomatosis during follow
175 up, suggesting mild combined immunodeficiency. Immune response modifier imiquimod or CO₂-
176 laser treatments had no effect. Papillomatosis paradoxically improved strikingly during the
177 mycophenolate treatment against anemia.

178

179 **Arthritis**

180 Patient III:1, with early-onset polyarticular juvenile arthritis, failed to tolerate or respond to various
181 medications summarized in the Supplement and Table 1. She also generated antibodies against
182 infliximab and golimumab. Finally, good response and tolerability were achieved with a combination
183 of azathioprine and high golimumab dose to block drug antibodies.

184

185 **DISCUSSION**

186

187 HA20 p.(Lys91*) disrupts regulation of multiple immunological pathways involving inflammation
188 and immunity. Patient's lung condition was most likely caused by inflammasome dysregulation. Lung
189 disease responsive to IL-1 antagonist anakinra and aphthous stomatitis alleviated with TNF- α
190 inhibitor etanercept together with our previously published results conform to abnormal NLRP3
191 inflammasome activity [9]. To our knowledge, this is the first description of monogenic inborn error
192 of immunity, in which anakinra has reversed severe pulmonary autoinflammation.

193

194 Atypical and treatment resistant anemia led us to consider a possibility of T cell-mediated
195 mechanisms and to test empiric and generally well-tolerated mycophenolate mofetil treatment. The
196 positive mycophenolate effect on anemia suggests a potential role for cytotoxic CD3⁺CD8⁺ T cells,
197 while failure to respond to IL-1 and TNF- α inhibition suggests mechanisms other than increased
198 inflammasome activity. HA20 also disrupts NF- κ B-mediated inflammasome regulation. Consistent
199 with more widely dysregulated NF- κ B due to p.(Lys91*), signs of aberrant B-cell development with
200 low switched memory B cells, elevated "immune-exhausted" C21^{low}CD38^{low} B cells, specific
201 antibody deficiency and production of autoantibodies were noted. These and the severe genital
202 papillomatosis suggest that HA20 may clinically cause mild combined immunodeficiency. The exact
203 etiopathogenesis and potential responses to mycophenolate need to be studied in larger cohorts of
204 HA20.

205

206 Our study was limited to a single rare A20 variant only and our experience should thus be interpreted
207 cautiously when planning treatment of HA20 patients with various mutations. However, coexistence
208 of multiple autoimmune conditions and infection susceptibility presenting in several organs is
209 unusual. Familial early onset autoimmune thyroid disease with growth delay, for example, should
210 lead to consideration of HA20. Lung inflammation, anemia and liver disease are also examples of
211 diversity of the A20 biological functions and the role in development of autoimmunity; it is possible
212 that HA20 disrupts signaling pathways even beyond the regulation of immunity. The widely
213 heterogenous phenotypes, diversity of immunological findings and unpredictable responses to
214 DMARD or bDMARD therapies signify an important clinical role for A20. We recommend that the
215 exact etiopathogenesis of HA20 caused by different *TNFAIP3* mutations should be further explored.

216 **Legend for Table 1.**

217 Summary of symptoms and age of onset of the symptoms associated with HA20 in the affected
218 family. Response to each treatment is summarized.

219

220 **Legend for Figure 1.**

221 A pedigree of the family with TNFAIP3 p.(Lys91*) (K91X) in three generations. Solid symbols show
222 the affected family members (positive for heterozygous for K91X mutation, +/-) described in detail
223 in the Supplement. White symbols show unaffected family members negative for K91X mutation (-
224 /-).

225

226 **Legend for Figure 2.**

227 Axial and coronal computed tomography (CT) scans of the chest demonstrates bilateral patchy
228 peribronchial nodular pulmonary opacities before treatment (A, B). After 1.5 months of anakinra (100
229 mg x 1 sc) treatment all the pulmonary lesions have disappeared completely (C, D). Histology of liver
230 shows moderate hepatosteatorosis (E, Hematoxylin-Eosin) and iron accumulation in the Kupffer cells
231 and sinusoidal macrophages (F, Prussian Blue).

232

233 **Declarations**

234 **Ethics approval:** The study has been approved by ethical committee of Oulu University Hospital.
235 Informed consent was obtained. The study was conducted according to the guidelines of Declaration
236 of Helsinki.

237 **Consent for participation:** Consent for participation was obtained.

238 **Consent for publication:** Consent for publication was obtained.

239 **Availability of data and material:** Not applicable

240 **Competing interests:** No competing interests.

241 **Funding:** Oulu and Helsinki University Hospital Research Funds.

242 **Authors' contributions:** TH, PV, AV, MS, MS, RK, NH, SK, PJ were responsible for treatment and
 243 follow up of treatment outcome. SK, KR, IP, VG, MV, KKE completed immunological analysis. HK,
 244 MM analyzed tissue histology. AJ completed radiological analysis. OK, JS completed genetic
 245 analysis. TH, KKE, MRJS were responsible for study design. All authors read and approved the final
 246 manuscript.

247 **Acknowledgements:** Not applicable

248

249 **References**

250

251

252 1. Zhou Q, Wang H, Schwartz DM, Stoffels M, Park YH, Zhang Y et al. Loss-of-function
 253 mutations in TNFAIP3 leading to A20 haploinsufficiency cause an early-onset
 254 autoinflammatory disease. *Nat.Genet.* 2016;48:67-73.

255 2. Aksentijevich I, Zhou Q. NF-kappaB Pathway in Autoinflammatory Diseases: Dysregulation
 256 of Protein Modifications by Ubiquitin Defines a New Category of Autoinflammatory
 257 Diseases. *Front Immunol.* 2017;8:399

258 3. Aksentijevich I, McDermott MF. Lessons from characterization and treatment of the
 259 autoinflammatory syndromes. *Curr.Opin.Rheumatol.* 2017;29:187-194.

260 4. Das T, Chen Z, Hendriks RW, Kool M. A20/Tumor Necrosis Factor alpha-Induced Protein 3
 261 in Immune Cells Controls Development of Autoinflammation and Autoimmunity: Lessons
 262 from Mouse Models. *Front Immunol.* 2018;9:104

263 5. Berteau F, Rouviere B, Delluc A, Nau A, Le BR, Sarabay Get al. Autosomic dominant
 264 familial Behcet disease and haploinsufficiency A20: A review of the literature.
 265 *Autoimmun.Rev.* 2018;17:809-815.

266 6. Aeschlimann FA, Batu ED, Canna SW, Go E, Gul A, Hoffmann P et al. A20
 267 haploinsufficiency (HA20): clinical phenotypes and disease course of patients with a newly
 268 recognised NF-kB-mediated autoinflammatory disease. *Ann.Rheum.Dis.* 2018;77:728-735.

269 7. Fischer JC, Otten V, Kober M, Drees C, Rosenbaum M, Schmickl M et al. A20 Restrains
 270 Thymic Regulatory T Cell Development. *J.Immunol.* 2017;199:2356-2365.

271 8. Zhang P, Wang PX, Zhao LP, Zhang X, Ji YX, Zhang XJ et al. The deubiquitinating enzyme
 272 TNFAIP3 mediates inactivation of hepatic ASK1 and ameliorates nonalcoholic
 273 steatohepatitis. *Nat.Med.* 2018;24:84-94.

9. Rajamäki K, Keskitalo S, Seppänen M, Kuismin O, Vähäsalo P, Trotta L et al. Haploinsufficiency of A20 impairs protein-protein interactome and leads into caspase-8-dependent enhancement of NLRP3 inflammasome activation. *RMD.Open.* 2018;4:e000740
10. Haapaniemi EM, Kaustio M, Rajala HL, van Adrichem AJ, Kainulainen L, Glumoff V et al. Autoimmunity, hypogammaglobulinemia, lymphoproliferation, and mycobacterial disease in patients with activating mutations in STAT3. *Blood* 2015;125:639-648.
11. Iwanaga N, Kolls JK. Updates on T helper type 17 immunity in respiratory disease. *Immunology* 2019;156:3-8.
12. Mulhern CM, Hong Y, Omoyinmi E, Jacques TS, D'Arco F, Hemingway C et al. Janus kinase 1/2 inhibition for the treatment of autoinflammation associated with heterozygous TNFAIP3 mutation. *J.Allergy Clin.Immunol.* 2019;144:863-866.

	Symptoms, age of diagnosis	Treatment, response
Patient 1	AITD, age not known	thyroid replacement therapy, responded
	Hepatitis, 43	no documented treatment
	Anemia, 43	no documented treatment
	Liver failure, 46	death
Patient 2	AITD, growth delay, 4	thyroid replacement therapy, responded
	Atrophic gastritis, 21	B12 substitution, increased serum B12 levels
	Psoriasis, 23	topical calcipotriol, corticosteroids and econazole, partial response
	Aphtous stomatitis	anakinra, no response canakinumab, no response etanercept, complete response
	Genital papillomatosis, 25	imiquimod, carbon dioxide (CO2) laser therapy, no response mycophenolate, complete response
	Episodes of oral and genital HSV, based on clinical presentation	acyclovir, partial response
	Pulmonary inflammation	broad spectrum antibiotics, no response prednisolone, no response anakinra, good response
	Anaphylactoid reactions, 28 - 31	prednisolone, good response
	Anemia, 30	prednisolone, good response, poor tolerance canakinumab, no response iv IG, no response etanercept, no response tofacitinib, poor tolerance, cytopenia

		mycophenolate, good response
	Hepatosteatosi Liver iron load, 30	no specific treatment
Patient 3	AITD, growth delay	thyroid replacement therapy, good response
	Anemia	no specific treatment
Patient 4	Cow's milk allergy	
	AITD, 1.6	thyroid replacement therapy, good response
	Juvenile idiopathic arthritis, 4.6	prednisolone and methotrexate, poor tolerance etanercept, poor response adalimumab and azathioprine, good response, poor tolerance tocilizumab and iv methylprednisolone, poor response adalimumab and methotrexate, partial response infliximab and methotrexate, good response, poor meth infliximab, insufficient response, infliximab antibodies anakinra, poor tolerance, uncertain response golimumab (recommended dose), leflunomide and response, golimumab antibodies golimumab (high dose) and azathioprine, good response

324
325
326

Hautala et al. Figure 2
Colour Image

327
328
329
330
331
332
333

334 Supplemental document

335 **Patient I:2.** Case description is based on hospital records. Since age 7, she (mother of II:1 and II:4)
 336 suffered from hypothyroidism treated with thyroid hormone replacement therapy. Between ages 30
 337 and 40, she experienced recurrent bone fractures due to osteoporosis, serum vitamin-D-25-OH
 338 concentration (<10 nmol/L, normal range 75 – 250 nmol/L) was low. Biopsies taken during
 339 gastroscopy due to low blood hemoglobin levels found atrophic gastritis. Age 43, she experienced a
 340 spell of unconsciousness. Brain computed tomography scan (CT) was unremarkable and her
 341 abdominal ultrasound was consistent with enlarged liver. Her liver enzymes were elevated: alanine
 342 transaminase 95 U/L (normal range 10 - 45), aspartate transaminase 284 U/L (normal range 15 - 35)
 343 and alkaline phosphatase 464 U/L (normal range 35 – 105). She was advised to avoid alcohol and
 344 medications with of liver toxicity. Age 46, she died of severe liver cirrhosis accompanied by weight
 345 loss and jaundice.

346 **Patient II:1.** Our index, a 30-year-old daughter of Patient I:2, had episodes of purulent otitis media
 347 and infectious pharyngitis in her childhood. At age 4, autoimmune hypothyroidism was noticed due
 348 to growth delay. Soon after an uncomplicated pregnancy (patient III:1) at age 21, she was found to
 349 suffer from atrophic gastritis and low vitamin B12 levels. At age 23, she developed skin psoriasis and
 350 psoriatic arthritis. At age 25, she developed treatment resistant genital papillomatosis and she suffered
 351 her first pneumonia. At age 28, she experienced an episode of prolonged oral and genital herpes,
 352 while also affected by genital papillomatosis. The diagnoses were based on clinical judgement.

353 At age 28 she was hospitalized due to fever and respiratory distress. Bacterial, mycobacterial, viral
 354 and fungal tests remained negative; antimicrobial treatments were ineffective, but she developed
 355 clostridial hemorrhagic colitis. For months after the acute phase, she suffered from severely
 356 incapacitating respiratory distress. Her chest high resolution computed tomography (HRCT)
 357 remained abnormal; findings suggested follicular bronchiolitis or bronchial lymphatic hyperplasia.

358 The combination of various complicated infectious, inflammatory and autoimmune diseases, and
 359 abnormal family history lead to basic immunologic testing with modest abnormalities (Supplement
 360 Table 1). Next, she and her family underwent whole exome sequencing (Rajamäki *et al*). A novel
 361 nonsense mutation in the exon 2 of *TNFAIP3*, c.A271T;p.(Lys91*)
 362 (ENSG00000118503:ENST00000433680) and functional testing confirmed haploinsufficiency of
 363 A20 (HA20) (Rajamäki *et al*). Consequently, autoinflammatory lung reaction was suspected.
 364 Administration of interleukin 1 antagonist anakinra led to rapid and complete resolution of her lung
 365 symptoms and radiologic abnormalities in chest HRCT (Figure 1, main document).

366 **Supplement Table 1.** Immunologic testing of patients.

	Patient II:1	Patient II:4	Reference
B-leukocyte count	$5.5 \times 10^9/\text{L}$	$6.5 \times 10^9/\text{L}$	$3.4 - 8.2 \times 10^9/\text{L}$
B-lymphocyte count	$1.7 \times 10^9/\text{L}$	$1.8 \times 10^9/\text{L}$	$1.2 - 3.5 \times 10^9/\text{L}$
CD19	$0.43 \times 10^9/\text{L}$	$0.35 \times 10^9/\text{L}$	$0.080 - 0.616 \times 10^9/\text{L}$
switched memory B cells	3.2% ↓	6.1 % ↓	6.5 – 29.2%
activated B cells (CD21 ^{low} CD38 ^{low})	13.7% ↑	15.7% ↑	0.8 – 7.7%
CD3	$1.30 \times 10^9/\text{L}$	$1.34 \times 10^9/\text{L}$	$0.742 - 2.750 \times 10^9/\text{L}$
CD4	$0.76 \times 10^9/\text{L}$	$0.76 \times 10^9/\text{L}$	$0.404 - 1.612 \times 10^9/\text{L}$
CD8	$0.50 \times 10^9/\text{L}$	$0.62 \times 10^9/\text{L}$	$0.220 - 1.129 \times 10^9/\text{L}$
Recent thymic emigrants (CD45 ⁺ CD62L ⁺ CD31 ⁺)	7.8% ↓	27.5%	14.4 – 38.3%
NK	$0.053 \times 10^9/\text{L}$ ↓	$0.13 \times 10^9/\text{L}$	$0.084 - 0.724 \times 10^9/\text{L}$
S-IgA	1.24 g/L	2.19 g/L	0.52 – 4.02 g/L
S-IgE	25 U/L	2 U/L	< 110 U/L
S-IgM	2.05 g/L	1.00 g/L	0.47 – 2.84 g/L
S-IgG	12.8 g/L	12.9 g/L	6.77 – 15.0 g/L
S-IgG1	10.9 g/L	-	4.05 – 10.11 g/L
S-IgG2	1.68 g/L	-	1.89 – 7.86 g/L
S-IgG3	0.60 g/L	-	0.11 – 0.65 g/L
S-IgG4	0.013 g/L	-	0.03 – 2.01 g/L
Anti-polysaccharide vaccine responses to Pneumovax®, of 10 tested serotypes*	2/10 ↓↓	1/10 ↓↓	

367 Tested routinely in an accredited laboratory. * for the used multiplex method see Selenius *et al*.

368 She developed treatment resistant anemia of unknown cause. The laboratory results, summarized in
 369 Supplemental Table 2, were not consistent with a known cause (*e.g.* hemolysis, inflammatory
 370 anemia). Though active reticulocyte production was seen in the bone marrow and the peripheral
 371 blood, in extensive hospital investigations and observation there was no proof of hemoglobin loss
 372 through feces, urine, exsanguination or intravascularly during her constantly worsening anemia.
 373 Finally, due to HA20, no evidence of B cell-mediated or other known causes of anemia and a theoretic
 374 possibility of T cell-mediated anemia, oral mycophenolate mofetil 500 mg x 2 – a generally well-
 375 tolerated drug suppressing T cell-mediated immunity – was empirically tested (see the main article
 376 and Supplemental Table 3).

377 **Supplemental Table 2.** Anemia-related testing in patient II:1.

	Patient II:1	reference
B-Hemoglobin	72 g/L	117 – 155 g/L
P-Hemoglobin	20 mg/L	< 50 mg/L
E-Mean cellular volume	85 fl	82 – 98 fl
B-Reticulocytes	$218 \times 10^9/L$ / 7.0%	$26 - 95 \times 10^9/L$ / 0.6-2.0%
P-Lactate dehydrogenase	175 U/L	105 – 205 U/L
P-Interleukin-2-receptor	276 U/L	160-620 U7L
P-interleukin-6	5.0 ng/L	< 7 ng/L
Coombs	negative	negative
S-Transferrin receptor	1.9 mg/L	0.8 – 1.8 mg/L
S-Ferritin	1055 µg/L	5 – 90 µg/L
S-transferrin	2.6 g/L	1.8 – 3.2 g/L
P-triglycerides	0.96 mmol/L	< 1.7 mmol/L
S-cobalamin	218 pmol/L	180 – 700 pmol/L
S-haptoglobin	0.78 g/L	0.29 – 2.0 g/L
S-erythropoetin	181 U/L	4.3 – 29 U/L
U-hemosiderin	negative	negative

382 **Supplemental Table 3.** Response of patient II:1 to selected treatments of anemia.
 383 The patient suffered from anemia of unknown cause as described in the main document. Panels A
 384 and B show blood hemoglobin concentrations (g/Liter) (A) blood reticulocyte counts ($\times 10^9/L$) (B)
 385 during daily prednisolone 10 mg of mycophenolate mofetil 500 mg x 2 dose. A lasting positive effect
 386 was observed, after the patient started receiving oral mycophenolate mofetil 500 mg twice daily.
 387 Weeks of treatment are shown in panels A and B. Panel C shows the effect of blood transfusions (red
 388 arrows, a 14-day time-period) and panel D shows the effect of weekly etanercept injections (black
 389 arrows, 1 month time-period) on blood hemoglobin concentration (g/Liter).

392

393 **Patient II:4.** Son of patient I:2 suffered from delayed growth and chronic rash and was diagnosed
 394 with autoimmune thyroid disease with positive thyroid peroxidase antibodies at age 12. At age 18, he
 395 developed mild anemia with blood hemoglobin 129 – 133 g/L (normal range 134 – 167 g/L) and
 396 elevated mean erythrocyte volume 98 – 104 fl (normal range 82 – 98 fl). There was no evidence of
 397 hemolysis and serum vitamin B12 concentration was normal (393 pmol/L, normal range 180 – 700
 398 pmol/L). He was free from any mucosal, pulmonary, joint or gastrointestinal symptoms. Although
 399 ocular findings are commonly seen in HA20, his ophthalmological analysis was unremarkable. He
 400 had normal absolute CD19+ B cell counts, but the percentages of his switched memory B cells were

401 decreased and of activated B cells increased, and he did not respond normally to antipolysaccharide
402 pneumococcal antigens (Supplement Table 1).

403 **Patient III:1.** The 11-year-old only child of patient II:1 was born after an uncomplicated pregnancy
404 with Cesarean section after 39 weeks of gestation. At age 2.5 months, she suffered from tearfulness,
405 eating problems, puking and vomiting. Brain MRI, neurological status and thyroid hormone levels
406 were normal. Symptoms were alleviated at age 6 months by elimination diet of cow's milk. Since age
407 2, she has become tolerant to cow's milk.

408 At age 1.4 y, stunted growth, constipation, poor weight gain and appetite lead to the diagnosis of
409 autoimmune hypothyroidism with elevated thyroid stimulating hormone (TSH, 98.7 mU/L, ref. 1.2 -
410 7.7 mU/L), low free thyroxine 9 pmol/L (ref. 10-25 pmol/L) and thyroid peroxidase antibody
411 positivity (> 600 U/L, normal <34 U/L). Transglutaminase IgA antibodies were negative. After
412 thyroid hormone replacement, catch up growth (-2.1 to -0.9 SD) was noted but she continued to suffer
413 from constipation.

414 At age of 4 y, she complained difficulties in using a pen and her foot appeared rigid when running.
415 Occasionally, she was found to drag her leg. Stiffness in the left wrist and both ankles were found.
416 She benefitted from non-steroidal anti-inflammatory medication. At age 4.6 y, the diagnosis of
417 polyarticular juvenile idiopathic arthritis (JIA) was set by a pediatric rheumatologist, with 15 to 25
418 inflamed joints. No signs of uveitis were found. Her complete blood count was normal and anti-
419 nuclear antibodies, cyclic citrullinated peptide antibodies and rheumatoid factor negative. The patient
420 was negative for HLA-B27, her serum IgA was normal, C-reactive protein was 15 mg/L, and
421 erythrocyte sedimentation rate only 9 mm/h.

422 She received corticosteroid injections into large joints, followed by three intravenous
423 methylprednisolone doses. The patient did not tolerate subcutaneous (s.c.) weekly methotrexate
424 (MTX) treatment. High dose (1mg/kg/week) anti-TNF monotherapy with etanercept was
425 subsequently used for 6 mo, with modest response. Anti-TNF- α therapy with adalimumab in

426 combination with azathioprine was discontinued despite good response, because of side effects. At
427 age 5 y, anti-IL6 therapy with tocilizumab (10 mg / kg, 190mg) once a month was started, combined
428 with oral prednisolone 7.5 mg every other day. Because of poor response to tocilizumab,
429 methylprednisolone pulses (30mg / kg) once a month were subsequently given. She refused to
430 continue her oral prednisolone with a daily dose of 2.5 mg.

431 At age 6 y, at least 35 inflamed joints were found and the patient was referred to our tertiary hospital
432 for intra-articular injections. White blood cell counts, immunoglobulin levels and complement
433 activity were normal. Subcutaneous MTX (10 mg / week) and adalimumab (20mg, every 2 weeks)
434 were started, adalimumab was later switched to intravenous infliximab (200mg, i.e. 8mg/kg) every 6
435 weeks due to challenges in subcutaneous administration. By age 7.4 y, JIA had become inactive while
436 the patient suffered from emesis related with MTX. MTX was discontinued at age 8 y. Her joint
437 symptoms increased 6 mo later, despite low ESR (11 mm/h) and high levels (405 AU/ml) of anti-
438 infliximab antibodies. With the found *TNFAIP3* mutation and functional testing (Rajamäki et al), the
439 diagnosis of HA20 was confirmed.

440 Based on her HA20 diagnosis, anakinra (70 mg, 2mg/kg) was tested at age 8.8 y. The patient
441 developed painful injection site skin reactions and after two weeks her treatment was stopped by her
442 family. Leflunomide (20 mg / every other day) in combination with golimumab (50 mg / month) and
443 oral prednisolone were then started, with gradually decreased dose down to 2.5 mg every other day.
444 At age 9, the patient became asymptomatic, without inflamed joints. At age 9.3 y, her joint symptoms
445 gradually returned, and active arthritis was found. She had developed neutralizing anti-golimumab
446 drug antibodies with her golimumab concentrations. Her dose was increased to 100mg/month
447 (2.6mg/kg) and azathioprine was gradually started (up to 2.2mg/kg/day). After that, joint symptoms
448 and drug antibodies have decreased and her polyarticular JIA has been mostly inactive.

449

450

451 **References for the supplement**

- 452 1. Rajamäki K, Keskitalo S, Seppanen M, Kuismin O, Vahasalo P, Trotta L et al.
453 Haploinsufficiency of A20 impairs protein-protein interactome and leads into caspase-8-
454 dependent enhancement of NLRP3 inflammasome activation. RMD.Open. 2018;4:e000740
- 455 2. Selenius JS, Martelius T, Pikkarainen S, Siitonen S, Mattila E, Pietikäinen R, et al.
456 Unexpectedly High Prevalence of Common Variable Immunodeficiency in Finland. Front
457 Immunol. 2017;8:1190.

458