**Abnormal differentiation of B cells and megakaryocytes in Roifman syndrome**

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**Abstract**

Background. Roifman syndrome is a rare inherited disorder characterized by spondyloepiphyseal dysplasia, growth retardation, cognitive delay, hypogammaglobulinemia and, in some patients, thrombocytopenia. Compound heterozygous variants in small nuclear RNA gene *RNU4ATAC*, necessary for U12-type intron splicing, were recently identified to drive Roifman syndrome. Objective. We studied three patients from two unrelated kindreds harboring compound heterozygous or homozygous stem II variants in *RNU4ATAC* to gain insights in the mechanisms behind this disorder. Methods. We systematically profiled the immunological and hematological compartments of the three Roifman patients and performed RNA sequencing to unravel important splicing defects in both cell lineages. Results. The patients exhibited a dramatic reduction in B cells, with differentiation halted at the transitional B cell stage. Despite abundant BAFF availability, development past this BAFF-dependent stage was crippled, with disturbed minor splicing of the critical MAPK1 signaling component. In the hematological compartment, Roifman patients demonstrated defects in megakaryocyte differentiation, with inadequate generation of proplatelets. Roifman platelets were rounder, with elevated tubulin and actin levels, and contained increased alpha and dense granule markers. Significant minor intron retention in 354 megakaryocyte genes was observed, including *DIAPH1* and *HPS1*, genes known to regulate platelet and dense granule formation, respectively. Conclusion. Together, our results provide novel molecular and cellular data towards understanding the immunological and hematological features of Roifman syndrome.

**Key Messages**

* Defects in the minor splicing complex drive a drive a defect in B cell differentiation leading to humoral immunodeficiency in Roifman syndrome
* Our findings highlight a critical role for minor intron splicing by U4atac during megakaryopoiesis and granulopoiesis

**Capsule summary**

In depth characterization of B cells and megakaryocytes provides novel insights in Roifman syndrome pathogenesis and reveals that most of the defects observed can be attributed to splicing abnormalities in small number of candidate genes.

**Keywords**

B cells, platelets, RNA processing, Roifman syndrome

**Abbreviations**

APRIL (a proliferation inducing ligand), BAFF (B cell activating factor), BCR (B cell receptor), BeviMed (Bayesian Evaluation of Variant Involvement in Mendelian Disorders), BLOC3 (biogenesis of lysosome-related organelles complex 3), CTCF (corrected total cell fluorescence), EM (electron microscopy), HSC (hematopoietic stem cells), IGF-1 (insulin-like growth factor), ITP (immune thrombocytopenia), IVIG (intravenous immunoglobulin), LSM (lymphocyte separation medium), MK (megakaryocyte), MOPD1 (microcephalic osteodysplastic primordial dwarfism type 1), MPV (Mean Platelet Volume), MSD (Meso Scale Discovery), PRP (platelet-rich plasma), RNAseq (RNA sequencing), ROUT (Robust regression and Outlier removal), snRNA (small nuclear RNA), SNV (single nucleotide variant), TAR (thrombocytopenia with absent radius), TPO (thrombopoietin),TSH (thyroid stimulating hormone)

**Introduction**

Roifman syndrome (OMIM#616651) is a rare disorder characterized by growth retardation, cognitive delay, retinal dystrophy, spondyloepiphyseal dysplasia and immunodeficiency1–4. Compound heterozygous variants in the small nuclear RNA (snRNA) noncoding gene *RNU4ATAC* have recently been found to cause Roifman syndrome5. This gene encodes U4atac, a snRNA which is indispensable for minor splicing and is responsible for removing U12-type introns6–8. The presence of U12-type introns is associated with genes involved in RNA processing and DNA repair9 but other processes can also be affected.

To date only ten cases with Roifman syndrome from seven pedigrees have been reported with causal variants in the *RNU4ATAC* gene5,10,11. Microcephalic osteodysplastic primordial dwarfism type 1 (MOPD1) (OMIM#210710), has also been associated with recessive variants in conserved regions of *RNU4ATAC*12,13.While described as clinically distinct conditions, the identification of causal variants in the same gene suggest that differential downstream effects drive the clinical differences between Roifman and MOPD1. MOPD1 patients in general seem to present with a more severe developmental phenotype than Roifman patients. Their main features consist of skeletal dysplasia, microcephaly with brain abnormalities and primary dwarfism. Only the retinal and immune phenotype seem more pronounced for Roifman syndrome patients, as retinal hypopigmentation and frequent infections have been described as part of MOPD114. Hence, it was hypothesized that mutations in MOPD1-associated regions of *RNU4ATAC* are of greater importance for minor intron splicing5, or, conversely, are required for the minor intron splicing of genes with more critical functions in development. Reported Roifman patients carry only one *RNU4ATAC* variant in either the critical region of the 5’ stem loop or (adjacent to) the Sm protein binding site, both considered as elements of crucial importance for splicing. The second variant is located in stem II, which has not been associated with MOPD15 (**Figure 1A**). Only recently, it was advocated that homozygous stem II variants are even sufficient to cause Roifman syndrome, pointing at a large impact of this region on the resulting patient phenotype10. The difference between alleles may also be related to differential downstream targets rather than to variant severity. Indeed, the different Roifman and MOPD1-implicated regions interact with different proteins, as the 5’ stem loop critical region mainly binds to NHP2L1 and PRPF31 and the stem II region interacts with NHPF4/PRPF3/PPIH15. This implies that the highly variable spectrum of both disorders is depending on the subset of genes affected (and their degree of minor intron retention).

The presence of clinically overt immunodeficiency in Roifman patients indicates an underlying dysfunction in the immunological compartment. Clinical work-up has previously revealed low numbers of circulating B cells and hypogammaglobulinemia1,2,5,10,11. At the hematological level, low platelet counts have been reported1,16. Potential explanations for these observations are aberrant XRCC5 splicing leading to defective B cell receptor (BCR) recombination5, and immune thrombocytopenia (ITP) as a cause of low blood platelet counts16. Here we have investigated the immunological and hematological systems of three Roifman patients from two unrelated kindreds bearing *RNU4ATAC* variants. Through coupled cellular biology and transcriptional analysis we identified key gene splicing defects capable of explaining the immune and blood phenotypes found in Roifman syndrome.

**Methods**

*Patient recruitment and genotyping*

Whole genome sequencing was performed for all three Roifman patients as part of the BRIDGE-BPD project. Details for the genetic analysis have been described previously17–19. Genes were next ranked using Bayesian Evaluation of Variant Involvement in Mendelian Disorders (BeviMed) which models the association between configurations of allele counts at rare variants in a locus and case/control labels19. Sanger sequencing of the *RNU4ATAC* gene was performed following amplification with primers 5’CTGTGGAGGCTGGAGGTAAGC3’ and 5’TTACTTGCGACACTAAAACCA3’.

*Flow cytometry*

Blood samples were collected in lithium-heparin tubes for separation of plasma and PBMCs using lymphocyte separation medium (LSM, MP Biomedicals). PBMCs were frozen in 10% DMSO (Sigma) and stored at −80 °C for a maximum of 10 weeks. Thawed PBMCs were stained with antibodies (**Supplemental Methods**). Foxp3 staining was performed after treatment with fixation-permeabilization buffer (eBioscience). Data were acquired on a BD FACSCantoII and analyzed with FlowJo (Tree Star). For kinetics assay, fresh isolated PBMCs were incubated in RPMI-1640 medium, supplemented with 10% human AB serum (Sigma-Aldrich), 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-Glutamine (all from Gibco, Life technologies). Recombinant human BAFF protein (R&D Systems) was used at 10 ng/mL.

*Serological assessment*

Circulating levels of BAFF were measured using human BAFF Quantikine ELISA (R&D Systems). APRIL was measured using human APRIL Quantikine ELISA (R&D Systems). Cytokine plasma concentrations were quantified by electrochemiluminescence immunoassay using the V-Plex human proinflammatory panel MSD (Meso Scale Discovery) plates. Samples and standards were prepared according to each manufacturer's instructions.

*NK cell assays*

NK cell assays were performed as previously described20, briefly NK cytotoxicity was measured in a 51Cr release assay. PBMCs were cultured with the NK-sensitive human K562 leukemia cell line (ATCC) that lacks expression of major histocompatibility complex class I. 51Cr labeled K562 cells were cultured for 4 hours with PBMCs (2.5-3 106 /mL) at a 50:1 effector:target cell (E:T) ratio, and the release of 51Cr was measured. Spontaneous and maximal release was determined by incubation of labeled target cells with medium or saponin (Merck), respectively. Specific lysis was calculated as follows: (experimental release – spontaneous release)/(maximal release – spontaneous release) x100. The degranulation capacity of NK cells was measured by the induction of CD107a surface expression after 2-hour incubation of PBMCs (0.2 106 /mL) with K562 cells (1:1). Perforin and granzyme expression was analyzed *ex vivo* by intracellular flow cytometry. For intracellular expression of IFNγ in NK cells by flow cytometry, PBMCs were cultured for 6 hours in the presence of K562 cells (1:1), with the addition of BD GolgiStop (1:150) and GolgiPlug (1:100) (both from BD Biosciences) for 5 hours.

*Functional platelet studies*

Platelet aggregation in response to different concentrations of multiple platelet agonists and ATP secretion were assessed via aggregometry as described21. In addition, flow cytometry was performed on PRP obtained from citrated blood. After stimulating platelets with different agonists for 10 min at RT, CD62P-PE was added to this suspension and incubated for 15 min in the dark prior to fixing (CellFix). Platelet number and size were also assessed on citrated whole blood using CD41a to identify platelets. Finally, dense granule functionality was investigated via a mepacrine (Q3251, Sigma, Saint Louis, Missouri, US) uptake/release assay22. Diluted PRP was incubated with different concentrations of mepacrine for 30 min in the dark, followed by activation of half of the sample with 50 μM TRAP for 15 min and sample fixation. Data were acquired on a FACSCantoII HTS and analyzed using FACSDiva software (all from BD, New Jersey, US).

*Platelet electron microscopy*

Peripheral blood was anticoagulated with trisodium citrate and centrifuged to obtain platelet-rich plasma (PRP). Samples were prepared as described23 and analyzed blindly using a transmission electron microscope (EM 900, Zeiss, Heidelberg, DE). Platelet morphology, size and alpha granules were further evaluated using ImageJ (National Institutes of Health, Bethesda, Maryland, US). Platelet shape and size were evaluated using 7000x EM images. The platelets were manually identified and platelet roundness was calculated as 4 × Area / (pi × Major axis2). The mean value per EM image (containing 5-15 platelets) was used for further analysis. Alpha granules were identified, measured and counted manually based on 20000x EM images. Alpha granule number and mean alpha granule size were quantified per platelet.

*Human CD34+ hematopoietic stem cell isolation and megakaryocyte differentiation*

Megakaryocte (MK) differentiation from peripheral blood-derived CD34+ hematopoietic stem cells (HSC) was performed as described previously17,24. In brief, HSC were cultured in StemSpan SFEM medium with StemSpan CC100 ensuring strong expansion of undifferentiated HSC for 3 days (Stem Cell Technologies, Vancouver, CA). Differentiation was initiated by adding 50 ng/ml thrombopoietin (TPO), 25 ng/ml stem cell factor and 10 ng/ml interleukin 1β (Peprotech, Rocky Hill, New Jersey, US). On day 6 of differentiation, 50 ng/ml TPO was resupplemented, resulting in proplatelet-forming MKs by day 11-12. MK were counted manually based on cell culture images which were divided in quadrants. MK were termed “proplatelet-forming” if they started to extend at least one protrusion.

*Immunostaining of megakaryocytes and platelets*

Day 12 differentiated MK were incubated on fibrinogen-coated coverslips for 3-4 hours at 37°C. Citrated PRP was left for 30 min at RT and next diluted 1/50 in Tyrode buffer. Platelets were fixed for 10 min in a final concentration of 4% PFA, before being spun onto poly-L-lysine-coated coverslips for 10 min at 600 G25. Attached cells were treated as described17,21,26 and incubated with antibodies (**Supplemental Methods**). MK were analyzed with a confocal microscope (AxioObserver.Z1, Zeiss, Heidelberg, DE) and platelet imaging was performed using a structured illumination microscope (Elyra S.1, Zeiss, Heidelberg, DE). Images were analyzed with ZEN Black (Zeiss, Heidelberg, DE) and ImageJ software (National Institutes of Health, Bethesda, Maryland, US). For the confocal images, cell surface area was determined based on the F-actin staining. Granule counting was performed on a default thresholded image via ‘Analyze particles’ after a median filter was applied to and subtracted from the original. Three random control images were used to optimize parameters for each staining which were then put into a macro for automated image analysis. MK were only included in DNA ploidy analysis if the number of nuclei was clear from either F-actin or granule staining. On the other hand, the raw 3D SIM images were processed to SR-SIM using the automatic algorithm of ZEN Black software, followed by maximum intensity projection. Resting platelets were selected on minimal size (> 2 μm) and circularity (> 0.7) for the alpha-tubulin staining and manually selected for the F-actin staining. Corrected total cell fluorescence (CTCF) was measured for both markers using the formula: Integrated Density – (Area of selected cell X Mean fluorescence of background readings). Three regions around the platelet were taken into account to calculate mean background fluorescence. Granule counting for platelets was performed in analogy with the MK analysis.

*Western blot analysis of platelets*

Platelet lysates were prepared as described17. Equal amounts of platelet lysates using 10 μg for alpha and 20 μg for dense granule markers (and DIAPH1) were evaluated using antibodies (**Supplemental Methods**). Immunoblots were analyzed using ChemiDocTM XRS+ (Bio-Rad Molecular Imager, Hercules, California, US) and Image Lab 5.2.1 software (Bio-Rad, Hercules, California, US).

*RNA sequencing and analysis*

Starting material for RNA sequencing (RNAseq) was either *ex vivo* FACS-sorted CD10+ cells or day 11 megakaryocyte cultures differentiated from HSC. Details for RNAseq and data analysis are in the **Supplemental Methods**. RT-PCR was performed on RNA of mature MK of all three patients to confirm minor intron retention in *DIAPH1* and *HPS1* with primers 5’GTTCACATCAGAAGTGAACT3’, 5’CCGTTCTTGTGCAGAACTAT3’ and 5’AGCACCTGGCTGGAGTTTAA3’, 5’CTTAGTTTTGACAAAGGCAG3’, respectively.

*cDNA and Intron retention analysis*

RNA was extracted with PAXgene Blood RNA tubes (PreAnalytix, Hombrechtikon, Switzerland) and cDNA was created with GoScript Reverse Transcription System (Promega, Wisconsin, US) according to manufacturer’s recommendations. PCR to confirm intron retention in MAPK1 and XRCC5 was performed with the following intron spanning primers: 5’TTCGAGCACCAACCATCGAG3’, 5’TCACAGGTGGTGTTGAGCAG3’, and 5’ATGGCACTGACAATCCCCTT3’, 5’TGTTTCATGTTGAATCACATCCAT3’, respectively.

*Statistics*

Statistical analysis was performed using PRISM software (GraphPad Software Inc., California, US). Healthy controls and Roifman patients were systematically analyzed as two groups using both-sided unpaired t-tests for the B cell experiments. Platelet and MK data were modelled and analyzed using several R packages (**Supplemental Methods**) after outlier removal using the Robust regression and Outlier removal (ROUT) method implemented in PRISM27.

*Study approval*

All individuals or their legal guardians gave informed consent prior to inclusion in the study. The study was approved by the Ethics Committee of UZLeuven (study numbers S52653 and ML3580). Patients were included in the multicenter NIHR BioResource-Rare Disease study for patients with diverse rare inherited diseases, including bleeding and platelet disorders and primary immune deficiencies.

**Results**

*Clinical presentation of Roifman syndrome in three patients with rare variants in RNU4ATAC*

We analyzed three patients that were clinically diagnosed with Roifman syndrome from two unrelated kindreds of Belgian European descent (**Figure 1B, Table I**). P1 and P2 were both born prematurely (32 weeks and 34 weeks of gestation) with low birth weights (-2.6SD and -2.1SD) and suffered from intrauterine growth retardation (**Supplemental Figure 1**). P3 was born at term with a birth weight of 2500g (-2,6SD) and length of 44cm (-3,6SD). All patients have moderate psychomotor delay and suffer from autism spectrum disorder. They were all diagnosed with retinal dystrophy with hypovascularisation (**Figure 1C**) and P3 suffers from eye motility problems. All manifest severe growth retardation. Skeletal X-rays revealed spondyloepiphysial dysplasia with irregularly shaped vertebral bodies with platyspondyly and flattened proximal femoral epiphyses in all patients (**Figure 1C**). In addition, all patients manifest pruritic ichthyosis-like skin rash, brachydactyly, hyperlaxity, hypotonia and hepatosplenomegaly. All patients have a clear endocrinological phenotype. Delayed puberty was common to all and P3 also exhibited hypogenitalism. Furthermore, P1 and P2 had minor elevations of circulating thyroid stimulating hormone (TSH) concentrations without clinical signs of hypothyroidism (**Table I**), whereas P3 was diagnosed with and treated for hypothyroidism in adulthood. Both P2 and P3 ultimately developed hypergonadotropic hypogonadism (**Table I**). Low levels of insulin-like growth factor (IGF-1) were detected. Finally, all patients present with absent adrenarche. Overall, P1 seems to display with a slightly more severe phenotype than his sister P2. P3 shows a milder phenotype than both siblings.

DNA samples from the three patients underwent whole-genome sequencing as part of a research project coordinated by the NIHR BioResource involving approximately 10,000 patients with rare diseases. The statistical association method for rare variants BeviMed19 was applied to patients with Roifman syndrome (using OrphaNet for labeling) and to controls i.e. unrelated probands not labeled with Roifman syndrome. The top-ranking locus corresponded to *RNU4ATAC,* which had anestimated probability of association equal to 1.000 with Roifman phenotypes. Co-segregation studies with Sanger sequencing confirmed the variants in the three patients and showed heterozygosity for the parents (**Figure 1, B and D**). For the first kindred, siblings P1 and P2 were compound heterozygous for single nucleotide variants (SNVs) c.16G>A in the stem II region and c.46G>A in the critical region of *RNU4ATAC* (**Figure 1, A and B**). Such combination is in line with published data and suports the hypothesis that the presence of stem II variants determines the differential presentation of Roifman orMOPD1 syndrome5. The SNV c.16G>A was also present in one of the 5 previously described Roifman pedigrees while c.46G>A was implicated in MOPD1 with reduced severity5. P3 is homozygous for stem II variant c.16G>A, making him the second patient with Roifman syndrome found to have this homozygous variant in the stem II region of *RNU4ATAC*10*.*

*High depth immune phenotyping identifies a block at the transitional B cell stage*

Immunological screening revealed that all patients have hypogammaglobulinemia and B cell lymphopenia (**Table I**). All patients suffer from recurrent viral infections, necessitating immunoglobulin substitution therapy. P2 suffered from mucocutaneous *Herpes simplex* infection and P3 presented a pneumococcal sepsis upon discontinuation of therapy. We further evaluated their immunological profile by studying 53 different parameters using a flow cytometry platform. Results showed that while T cell subset numbers and associated cytokines levels were within a normal range (**Supplemental Table I**), circulating B cells (CD19+) and naïve B cells (CD19+ CD24+ CD38+) were significantly lower than normal values (**Figure 2, A and B**). We analyzed other B cell subsets and, by contrast, the percentage of transitional B cells (CD19+ CD24hi CD38hi) (**Figure 2C** and **Supplemental** **Figure 2** for gating strategy) was significantly increased over the healthy controls, indicating that B cells differentiation up to the transitional B cell stage was intact, however further maturation/survival was arrested.

To determine the mechanism of transitional B cell blockade we performed RNA sequencing on CD10+ B cells (**Supplemental Worksheet 1**). Differential expression analysis identified splice factors and small nuclear RNA (snRNA) as the primary enrichment (**Supplemental Table II, Supplemental Figure 3**), indicating compensatory upregulation following defective minor intron splicing. Of the 881 genes that contain a U12 intron28, 178 expressed genes had minor intron retention within patient transitional B cells (**Supplemental Worksheet 2**). *XRCC5* has a U12 intron (ID 28319)28, and based on its function in repairing any DNA double-stranded breaks introduced during V(D)J recombination29, defective XRCC5 splicing has been proposed as a cause of the B cell deficiency in Roifman5. Within transitional B cells, the U12 intron of XRCC5 was retained in P1, P2 and P3 (**Supplemental Figure 4**). We therefore assessed the BCR repertoire in both patients, however there was no alteration in V(D)J recombination (**Figure 2, D and E**). Furthermore, we evaluated the median CDR3 length of IGH rearrangements, which did not deviate from the healthy controls (**Figure 2, F and G**). Together, the stage of B cell differentiation blockade and the presence of normal BCR recombination demonstrate that the B cell deficiency is not due to defective BCR rearrangement, but rather it is at a later stage of B cell maturation.

*BAFF signaling pathway implicated in transitional B cell blockade*

Early B cell differentiation is independent of BAFF (B-cell Activating Factor), while mature B cells depend on BAFF and its interaction with BAFF-R30,31. The transitional B cell stage marks the point at which B cells become dependent on BAFF-R, with naive B cells binding BAFF exclusively through BAFF-R32,33. Baff-deficient mice manifest B cell deficiency from the transitional stage onwards30,34, indicating the BAFF pathway as the only known biological pathway which would replicate the Roifman phenotype. To assess if BAFF expression was deficient in Roifman patients, we measured plasma BAFF levels by ELISA, in both healthy controls and patients. In line with previous work11, Roifman patient’s BAFF levels were significantly elevated compared to healthy controls (**Figure 3A**). A proliferation-inducing ligand (APRIL), can also promote the proliferation and differentiation of B cells, signaling through TACI, was not altered (**Figure 3B**). BAFF-deficiency could therefore be excluded, with the elevation accounted for by decreased consumption owing to the B cell lymphopenia (observed also in patients on B cell depletion therapy, data not shown).

We next assessed BAFF-R expression in transitional B cells in both patients and controls. BAFF-R extracellular MFI expression in transitional B cells (CD19+ CD24hi CD38hi BAFF-R+), was lower than in controls (**Figure 3C**). At an mRNA level, BAFF-R was unchanged (**Supplemental Worksheet 1**), and splicing was not altered. As BAFF receptor may be internalized in the cell membrane, we performed an intracellular staining; however, no major intracellular compartment was observed (data not shown). We therefore tested the possibility that decreased BAFF-R expression was downstream of chronically elevated BAFF levels. For the Roifman patients there was an inverse correlation found between BAFF and BAFF-R, with lowest BAFF-R expression on the B cells of patients with the highest concentration of circulating soluble BAFF (**Figure 3D**). Healthy control (n=3) and patient (n=3) PBMCs were cultured in BAFF-free media (CRPMI) and in media containing recombinant BAFF protein. We observed that patients PBMCs cultured in CRPMI start to recover BAFF-R extracellular expression, and, conversely, healthy control B cells incubated with recombinant BAFF protein start to downregulate BAFF-R expression to levels observed in patients (**Figure 3E**). These results indicate that the reduction in BAFF-R in patient B cells is secondary to the chronic elevation of BAFF, and does not drive the defect in transitional B cells.

Ingenuity pathway analysis of the 178 expressed genes with minor intron retention (**Supplemental Worksheet 2**) identified MAPK1 within defined major B cell signaling pathways. A crucial mechanism through which BAFF promotes B cell survival is the downregulation of BIM, which is achieved by sustained activation of MAPK1. MAPK1 signaling phosphorylates BIM, promoting the ubiquitination and degradation of this pro-apoptotic factor33,35. BAFF-dependent survival of transitional B cells thus relies on MAPK1 signaling. In Roifman patients, we observed a defect in the removal of the minor intron (intron 2) in MAPK1 **(Figure 3F and G)**. Intron retention introduces a stop codon in the reading frame that can account for the protein being truncated **(Figure 3H)**.MAPK1 mRNA expression was moderately reduced in Roifman patients **(Figure 3I)**, however, only 47% and 48% of the mRNA transcripts copies, of P1 and P2 respectively, were correctly spliced **(Figure 3J)**.Surviving B cells demonstrated a compensatory upregulation in MAPK1 protein expression, however the concordant upregulation of the obligate MAPK3 heterodimer partner resulted in a skewed ratio of MAPK1:MAPK3 (**Figure 3K**), potentially driving the observed disturbance in the cellular phenotype. This defect intheMAPK1-MAPK3 complex, and the altered BAFF-BAFF-R relationship, provides a potential mechanistic explanation for the failure of B cell differentiation at the transitional stage, as the anti-apoptotic pathway of BAFF-R would be specifically impaired, resulting in a failure of survival and further maturation into the naïve B cell state.

*NK cell defects in Roifman patients*

As an independent immunological disturbance, NK cells were also reduced in Roifman patients (**Supplemental** **Figure 5A**), with a skewing towards the CD5bright population (**Supplemental** **Figure 5, B and C**). We further evaluated their cytotoxic functions by 51Cr-release and a CD107a degranulation assay against K562 tumor cell line. We observed that the percentage of 51Cr-release is reduced in all the patients when compared to the controls (**Supplemental** **Figure 5D**). The same tendency was not observed in the CD107a surface cell expression (**Supplemental** **Figure 5, E and F**), indicating a specific defect in cytotoxity rather than degranulation. Analysis of the expression of perforin and granzymes in NK cells indicated that granzyme A, granzyme B and perforin were decreased in the patients, while granzyme K levels were comparable to the controls (**Supplemental** **Figure 5, G-J**). Defective NK function may act as an independent factor contributing towards some aspects of the immunodeficiency identified in Roifman patients.

*Platelet number, size and function in Roifman patients*

Roifman patients presented with a drop in platelet count during clinical follow-up and remained mildly thrombocytopenic upon reaching adolescence (**Supplemental** **Figure 6, Table I**). Such an age-related decrease in platelet count is not typical for ITP which is also defined as having platelet counts lower than 100 x 109/L36.The Mean Platelet Volume (MPV) was within normal limits, except for patient P1 with mostly undetectable MPV values (**Table I**). Flow cytometry was performed on whole blood from P1 and an age- and gender-matched healthy control. No difference in platelet size could be detected between the patient and control, but P1 did have reduced platelet numbers (**Supplemental Figure 7**). Platelet ATP secretion and aggregation responses to different concentrations of collagen, ADP and epinephrine were only reduced when platelet counts in platelet-rich plasma were lower than 250 x 109/L that is typically used for functional testing (**Supplemental Table III**). To avoid this effect of platelet count on functional testing, flow cytometry detecting P-selectin (CD62P) release from alpha granules after platelet activation was performed for P1 (**Supplemental Table III**). Relative to an age- and gender-matched control, P1 responded normally to the agonists and even had slightly elevated CD62P expression (**Supplemental Figure 8**). Dense granule uptake and release using the mepacrine assay was normal for platelets of patient P1 (**Supplemental Table III**). Despite the fact that platelets of all three patients with Roifman syndrome are reduced in number, platelet functions are normal and the patients never presented with any obvious or trauma-related bleeding.

*Altered platelet morphology and granulation in Roifman patients*

Platelet morphology was studied by electron microscopy (EM) for patients P2 and P3 and platelet shape, size and granules were compared to age- and gender-matched controls. EM revealed that platelets of Roifman patients were more round instead of discoid (**Figure 4, A and B**). Because of this roundness, platelets of Roifman patients also appeared to be larger when evaluating EM slices (**Figure 4, A and C**). However, both the MPV values and flow cytometry data have demonstrated that platelet volume is indeed normal. The presence of several enlarged alpha granules was noticed (**Figure 4D**) and quantification revealed a slightly higher average alpha granule size in Roifman patients though this was not significant. Alpha granule number was also normal (**Figure 4E**). In addition, a blinded pathologist described numerous dense granules in the platelets of Roifman patients P2 and P3. Immunostaining for dense granule and lysosome marker CD63 on platelets of P3 also pointed to a significantly higher number of these organelles in comparison with a healthy control (**Figure 4, F and G**). Western blot analysis of total platelet lysates showed increased levels of platelet alpha granule-stored proteins VWF and TSP1, relative to platelet receptor integrin ß3 (**Figure 4H, Supplemental Figure 9A**). In contrast to the normal dense granule mepacrine uptake and release and normal ATP secretion after platelet activation, Roifman platelets express increased levels of CD63 and LAMP2 as detected by Western blot (**Figure 4H**, **Supplemental** **Figure 9B**). This would mean that platelets of patients with Roifman syndrome have increased numbers of dense granules with a normal function. Additionally, a few rounder platelets of patients with Roifman syndrome showed granule centralization and empty vacuoles were detected by EM (**Supplemental** **Figure 10**).

*Defective megakaryopoiesis for Roifman patients*

As Roifman patients have a reduced number of platelets with morphological defects, *in vitro* megakaryopoiesis was studied using peripheral blood-derived CD34+ hematopoietic stem cells (HSCs) from the three patients and age- and gender-matched healthy controls. MK at day 12 of differentiation contained more immature small MK cells for all Roifman patients and a mild though significant reduction in proplatelet formation (**Figure 5, A and D**). MK from patients P1 and P2 were used for RNAseq and MK from patients P1 and P3 for detailed morphological studies. Similar to healthy controls, MK of patients with Roifman syndrome underwent endomitosis, which resulted in a very large and lobulated nucleus. The maturing MK were evaluated grouping cells on similar DNA ploidy levels to follow-up granule formation and cytoplasmic enlargement during this process. Immunostaining using alpha granule marker VWF (**Figure 5B**) showed a significantly lower increase in alpha granule number during MK maturation for Roifman patients compared to healthy controls (**Figure 5E**). In contrast, only a mild though not significant decrease in dense granule number (using CD63 as marker) was observed in the maturing Roifman MK (**Figure 5, B and F**). Moreover, F-actin immunostaining revealed that the cytoplasmic fraction of the patient MK remained smaller during this process (**Figure 5, C and G**). As MK of Roifman patients appeared immature and were reduced in size, it was expected that granule formation would also be impaired. The strong decrease in alpha granule number together with the small MK cell size results in a normal total alpha granule number in platelets. Dense granule number on the other hand is only slightly lower in the MK of patients with Roifman syndrome, generating platelets with slightly elevated numbers of dense granules.

*RNAseq of Roifman megakaryocytes showed aberrant minor intron retention*

RNA sequencing was performed on differentiated MK from patients P1 and P2 and two age- and gender-matched controls, in duplicate cultures for each. Roifman MK clearly clustered apart from controls on the basis of gene expression profiles (**Supplemental** **Figure 11**). Differential exon/intron usage analysis identified 1390 alternatively used exon/intron bins between the two groups (with FDR <0.05 and estimated fold change >1.5) (**Supplemental Worksheet 3**). Of the 300 most significantly differing bins, 76% was an exact match to or overlapped with minor intron regions, confirming MK are among the affected cell types in Roifman syndrome. Aberrant minor intron splicing was thus found for 354 different genes, with organelle organization as most enriched Gene Ontology term associated (**Supplemental Table IV**). Interestingly, we detected highly significant intron retention in *DIAPH1* (*P* = 1.49x10-127), *HPS1 (P =* 5.95x10-56*)* and *RASGRP2* (*P* = 9.43x10-173), three genes known to be involved in platelet disorders26,37,38 (**Supplemental Worksheet 3**).

Confirmation studies were performed for platelet and granule formation genes *DIAPH1* and *HPS1,* respectively*.* Sashimi plots show the location and extent of intron retention in patient MK samples (**Figure 6A**). RT-PCR analyses covering these regions confirmed that the minor introns are retained in MK from P1, P2 and P3, while these alterations are not present in healthy controls (**Figure 6B**). Most interestingly, DIAPH1 is a cytoskeleton regulator which stimulates actin assembly and stabilizes microtubules39. Only recently, Stritt *et al*.26 showed the importance of these processes forplatelet formation as well as platelet cytoskeletal organization. They demonstrated that merely small changes in *DIAPH1* activity can cause macrothrombocytopenia, by investigating platelets of patients with a dominant gain-of-function variant. We therefore performed additional experiments to study the platelet cytoskeleton in P1 and P3. Immunostaining for alpha-tubulin showed a normal distribution of microtubules, but an increase in microtubule levels (**Figure 7A**). Moreover, F-actin was elevated in platelets of patients with Roifman syndrome, which implies upregulation of actin polymerization (**Figure 7B**). As our findings in patients with Roifman syndrome were milder, but reminiscent to those in patients with *DIAPH1* gain-of-function26, we also evaluated protein expression. Western blots analysis of platelet lysate reveals a strong increase in DIAPH1 protein levels in Roifman patients (**Figure 7C**). A feedback loop in response to incorrectly spliced RNA could explain this overcompensation, as was previously suggested to be the case for 30% of the affected genes in this disorder5, though this requires further confirmation studies.**Discussion**

We here provide novel insights in the immune deficiency and thrombocytopenia reported in patients with Roifman syndrome by deep phenotyping and RNA sequencing of B cells and megakaryocytes of three patients from two unrelated kindreds that have causal variants in *RNU4ATAC*. In line with published data5,11, patients P1 and P2 have one variant in the stem II region, which base pairs with another noncoding RNA for proper minor spliceosome loading, while the second variant is located in the *RNU4ATAC* critical regions implicated in the severe syndrome MOPD112,13. Previously, Roifman syndrome siblings have been reported where the sister showed an overall milder phenotype than the brother, indicating skewed X-inactivation and possible discrepancy in intron retention could be present2,11. In our study, while we observe this trend is upheld, albeit to a minor level, in the clinical phenotypes, although systematic differences were not observed at the molecular level. Additionally, our study expands the known genetic space of Roifman mutations by describing a homozygous patient (P3) with a *RNU4ATAC* stem II variant, confirming that the disease can occur without the presence of a variant in MOPD1-implicated regions of the *RNU4ATAC* gene. In 2015, it was already postulated that the differential presentation of MOPD1 and Roifman syndrome was a consequence of presence of a stem II variant5, as no MOPD1 patients have ever been reported with a variant is this domain. Now we can elaborate on this by verifying that *RNU4ATAC* variants in the stem II region are sufficient to cause the full spectrum of Roifman syndrome without the presence of mutations in the MOPD1 critical region11. Although all features of this disorder are present, P3 does present with a milder phenotype when compared to compound heterozygous Roifman patients (P1 and P2), and is closer to the control population in several cellular assays performed. These findings can potentially be explained by the location of the variant, where the region can withstand several mutations and retain function as long as pairing with U6atac snRNA is preserved40. We could therefore speculate that the disturbance of RNU4ATAC function, caused by variants in MOPD1-implicated regions, worsens the phenotype of Roifman syndrome. Moreover, the distinct presentation of both disorders could be a consequence of variation in the subset of strongly affected genes and of severity of intron retention. Further splicing studies on both MOPD1 and Roifman patients would however be needed to fully characterize the downstream effects of variants in the different regions of the *RNU4ATAC* gene*.*

The most striking immunophenotypic finding in Roifman patients is the paucity of naïve B cells, accompanied by hypogammaglobulinema and clinical susceptibility to infections. This defect lead to speculation by Merico *et al.*5 that intron retention in the *XRCC5* gene leads to defective generation of the BCR repertoire, a process which would cause blockade of B cell differentiation at the pre-/pro-B cell stages in the bone-marrow. Here we formally exclude this possibility through two independent lines of evidence: first, the normal appearance of transitional B cells, not observed in mice or patients with defects in BCR recombination; and second, direct measurement of a normal BCR repertoire in patients. Instead, our cellular and molecular characterization of Roifman patient B cells identifies a latter stage of B cell differentiation as the source of immunodeficiency. The dramatic fall off in B cell numbers past the transitional B cell stage implicates defects in the BAFF pathway in Roifman patients.

The transitional B cell stage marks the point at which B cells become dependent on BAFF-R for survival35,41, with MAPK1 signaling required to counter the pro-apoptotic function of Bim in transitional and naïve B cells41. Defects in Baff in mice phenocopy the B cell differentiation pattern observed in Roifman patients31,41, providing a potential mechanistic basis for the cellular phenotype. BAFF was highly abundant in Roifman patients, thus reducing BAFF-R expression and owing to the B cell immunodeficiency42. An independent molecular analysis identified MAPK1 splicing as a major impairment in Roifman patients, with fewer than half mRNA transcripts showing correct splicing over the exon 2-3 minor intron boundary. Despite compensatory mechanisms reducing the disparity at the protein level, this defect lead to distorted ratios of the constituent components of the MAPK1-MAPK3 heterodimer signaling complex. With MAPK1-MAPK3 heterodimers being required for the pro-survival function of BAFF signaling, this defect provides a sufficient explanation for observed phenotype, although it does not exclude the presence of other B cell-intrinsic defects in differentiation and survival owing to loss of the minor splicing complex. Notably, defects at this later stage of B cell differentiation may provide a unique therapeutic target for the immunodeficiency component of Roifman syndrome. While BCR recombination defects can only be treated through bone-marrow transplantation, a problematic solution for syndromic patients, the molecular analysis presented here suggests that peripheral agonism of MAPK1 or antagonism of BIM would be sufficient to rescue the survival of transitional B cells and allow the differentiation of a fully functional naïve B cell pool.

The three reported patients also show a slightly reduced number of platelets yet with normal function. Previously, it was proposed that thrombocytopenia in Roifman syndrome is immune mediated, as in ITP. In 2003, Roifman and Melamed16 reported on one case that responded well to intravenous immunoglobulin (IVIG) treatment. However, another Roifman patient responded poorly to IVIG. Our findings revealed MK and platelet defects in patients with Roifman syndrome, elucidating that the observed mild thrombocytopenia is inherent to the disorder and not just secondary to the immunological defects. Moreover, 354 genes in Roifman MK undergo aberrant splicing and it is expected that such defects modify megakaryopoiesis, especially since most inherited thrombocytopenias are dominant as result of only a single gene variant43. EM analysis, immunostainings and Western blots showed rounder platelets that contain slightly more dense granules and some enlarged alpha granules. Alpha and dense granule markers were strongly increased. Such defects are not typical for ITP that results from increased removal of antibody-coated platelet by the spleen. We found that HSC of Roifman patients undergo abnormal megakaryopoiesis with reduced proplatelet formation, have smaller MK due to aberrant cytosol maturation and as a consequence have decreased alpha granule formation during MK maturation. This would implicate that snRNA U4atac regulates splicing of genes during megakaryopoiesis. The importance of RNA processing and small RNAs for megakaryopoiesis can be expected based on a number of studies44–48, but its role in pathology is unknown. The only other syndrome that points to the importance of correct RNA processing in megakaryopoiesis is thrombocytopenia with absent radius (TAR) syndrome49. Compound heterozygous variants are present in *RBM8A*, which encodes a subunit of the exon-junction complex involved in nuclear export and localization of specific transcripts, translation and nonsense-mediated RNA decay. It is however still unknown how defects in this pathway affect megakaryopoiesis in TAR syndrome.

We hypothesize that defects in minor splicing affect multiple genes that are important for megakaryocyte and platelet function and that they each could contribute to the overall phenotype. Significant minor intron retention was detected in 354 genes, among which two genes previously associated with platelet phenotypes comparable to those observed in Roifman patients, being *DIAPH1*26and *HPS1*37. The technical and biological replicate RT-PCR confirmed intron retention for both *DIAPH1* and *HPS1* transcripts. Interestingly, a dominant gain-of-function variant in *DIAPH1* was recently reported to cause macrothrombocytopenia with normal platelet function though abnormal morphology26. These patients have reduced proplatelet formation in MK assays and EM of their platelets showed enlarged platelets with some giant alpha granules. Aberrant tubulin and actin levels were also noticed in immunostainings of platelets, all remarkably similar as found for the patients with Roifman syndrome. The dominant p.R1213\* DIAPH1 variant resulted in a premature stop located in the diaphanous autoregulatory domain which interacts with the diaphanous inhibitory domain to limit DIAPH1 activity. The observed intron 11-12 retention would result in a premature stop leading to mRNA decay or a truncated shorter protein in the middle of the dimerization domain (**Supplemental Figure 12A**). Western blot analysis did not reveal the presence of a smaller truncated protein, but rather enhanced DIAPH1 expression. We therefore hypothesize that the presence of incorrectly spliced mRNA leads to a strong increase in *DIAPH1* transcription via a feedback loop. Such an overcompensation mechanism was previously suggested for other genes studied in Roifman syndrome5. Recessive variants in *HPS1* are known to cause Hermansky-Pudlak syndrome50,51, where multiple patients’ platelets showed dense granule defects, indicating its central role in the formation of these platelet organelles50. HPS1 is a component of the biogenesis of lysosome-related organelles complex 3 (BLOC3) and is thought to play a role in coordinating the transport of cargo into developing granules51. Our Roifman patients show the opposite dense granule phenotype, as their platelets contain a slightly higher number of dense granules and have increased storage of dense granule protein. The minor intron retention observed in *HPS1* in Roifman MK is located in one of two HPS1 regions that interacts with HPS4 (**Supplemental Figure 12B**), another BLOC3 component52. Our findings indicate that minor intron retention might enhance or stabilize HPS1 and HPS4 interaction, and therefore also promote BLOC3 function, though additional studies are required to support this.

In conclusion, we describe two patients with compound heterozygous variants and one with a homozygous variant located in stem II and presenting a milder phenotype. We identified the transitional B cell as the key cell type affected by loss of minor intron splicing, with a blockade in further differentiation despite the presence of a normal BCR repertoire. Defective MAPK1 minor intron splicing is sufficient to explain this defect, with the known biological role in coupling BAFF signaling to transitional B cell survival. At the hematological level, we identified defective megakaryocyte differentiation as the most likely cause for the observed thrombocytopenia, with defective splicing of *DIAPH1* and *HPS1* potentially explaining the reduction in platelet formation and presence of abnormal granules. In all, our results provide new insights in the role of minor intron splicing in the hematopoietic and the immune system, mechanistically explain key phenotypic features, and identify valuable therapeutic intervention targets for clinical management of aspects of Roifman syndrome.

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**Figure Legends**

**Figure 1. Compound heterozygous and homozygous mutations in the RNU4ATAC gene cause Roifman syndrome.**

(A) Graphical representation of the U4atac RNA secondary structure upon binding to U6atac (grey). Known MOPD1-implicated variants are shown in red and previously described Roifman-causing variants (orange) are compound heterozygous for one variant in a MOPD1-region and a second in stem II or homozygous for a variant in the latter domain. Such compound heterozygous variants are also found for P1 and P2, while P3 is homozygous for a stem II variant (green). (B) Pedigree of three Roifman patients (P1-3) from two kindreds (K1-2) showing the segregation of RNU4ATAC variants. (C) Clinical images representing the (i, ii) retinal phenotype of P1 and P2; respectively. Optic disc are pale, white arrow shows narrowed blood vessels and black arrow shows macular edema. Retinal dystrophy is less marked in P2 than in P1 (iii-v). Skeletal radiographs showing flattened, biconvex vertebrae and mild osteopenia (P1), flattening and broadening of proximal tibial epiphysis with flattened eminentia intercondylaris (P1) bilateral dysplastic proximal epiphyses of femora (P3). (D) Sanger sequencing. Kindred 1: P1 and P2 inherited the c.G16A allele from the father and the c.G46A allele from the mother. Kindred 2: P3 is homozygous for the c.G16A variant and both his parents are heterozygous.

**Figure 2. B cell differentiation is terminated at the transitional B cell stage in Roifman patients.**

(A) Percentage of B cells (CD19+) (B) Percentage of naïve B cells (CD19+CD24+CD38+). (C) Percentage of transitional B cells (CD19+CD24hiCD38hi) (D) Percentage of rearrangements for V gene and (E) J gene usage in purified CD10 positive cells. (F) Median CDR3 length of IGH rearrangements. (G) Pielou’s evenness index. \* p<0.05 \*\*p< 0.005 \*\*\*\*p<0.00001.

**Figure 3. Defects in MAPK1 in the BAFF signaling pathway in Roifman syndrome B cells.**

(A) Circulating plasma BAFF concentration in patients and controls. (B) Circulating APRIL concentration in plasma between patients and controls. (C) BAFF-R MFI of transitional B cells (CD19+CD24hiCD38hi). For healthy controls, box and whiskers represent ranges of 25 to 75% and 5 to 95% (n=29 or n=9). For patients, SEM is shown. (D) Correlation of circulating BAFF and BAFF-R MFI expression in healthy controls (n=9) and Roifman patients (n=3). (E) BAFF-R MFI on PBMCs of healthy controls (mean n=3) and Roifman patients, after a short incubation period (15 min and 60 min) with BAFF-free media (CRPMI) or media containing recombinant BAFF. (F) Sashimi plot showing MAPK1 intron retention (IR) between exon 2 and 3 in P1 and P2. (G) PCR on cDNA of healthy controls (HC), and all patients showing intron retention (1821bp) between exon 2 and exon 3. (H) (i) MAPK1 intron retention between exon 2 and 3 red arrow showing the first stop codon introduced by retained intron. Correct transcript has a total of 9 exons, 8 coding exons and a transcription length of 360 residues. (ii) Relative introns size (intron retained in orange). (I) MAPK1 gene expression. (J) Percentage of MAPK1 mRNA transcripts with correctly splicing at the exon 2-3 boundary. (K) Flow cytometry histograms for MAPK1 and MAPK3 expression on transitional B cells, and the resulting MAPK1:MAPK3 ratio. SEM is shown. Unpaired t-test was used \*p< 0.05.

**Figure 4. Aberrant platelet morphology in Roifman syndrome.**

(A-C) Representative EM of platelets at 7000x. Platelet size and roundness were calculated in four healthy controls vs. P2-3. Scale bar: 1,50 μm. (D-E) EM images at 20000x were used for the evaluation of alpha granule size and number per platelet in the same subjects. Arrows indicate representative examples of alpha granules. Scale bar: 0,50 μm. Linear mixed effects models were fitted to the logit of platelet roundness, the log of platelet size and the log of mean alpha granule size with gender and genotype as fixed effects and individuals as random effects. Analogously, a negative binomial mixed effects model was fitted for platelet alpha granule number. (F-G) Immunostaining of poly-L-lysine immobilized platelets for CD63 and F-actin. Dense granule number was assessed per resting platelet for one healthy control vs. P3. Scale bar: 5 μm. A negative binomial regression model was fitted for dense granule number modelling genotype as fixed effect. (H) Western blot analysis on platelet lysate showing residual protein levels (accounting for the technical repeats) of alpha granule markers vWF and TSP1 and dense granule (and lysosome) markers CD63 and LAMP2 (three repeats of two healthy controls vs. P1-2-3). The log of the normalized intensity of each granule marker was modelled using linear regression accounting for genotype and experiment. In all graphs, box and whiskers represent ranges of 25 to 75% and 5 to 95%.

**Figure 5. Megakaryocyte differentiation defects in Roifman patients.**

(A) Representative cell culture images of day 12 differentiated MK, showing proplatelet formation in healthy controls. (B) Immunostaining of MK on day 12 using alpha granule marker vWF or dense granule (and lysosome) marker CD63 together with F-actin. MK of the same ploidy are shown allowing the comparison of granule formation. Scale bar: 20 μm. (C) Different stages in MK maturation are depicted using immunostaining of CD63 and F-actin to demonstrate cytoskeletal changes. Scale bar: 20 μm. (D) Proplatelet formation was evaluated based on cell culture images (three healthy controls vs. P1-2-3). A linear mixed effects models was fitted to the proportion of proplatelet-forming MK with the experiment and genotype as fixed effects and the individuals as random effects. (E) The rate of alpha and (F) dense granule formation was evaluated in maturing MK by diving them in ploidy groups. Differences in slope were investigated based on two differentiations of a healthy control vs. P3 and one differentiation of two healthy controls vs. P1. (G) The cytoplasmic enlargement was evaluated in these MK using F-actin staining. In all graphs, box and whiskers represent ranges of 25 to 75% and 5 to 95%. Negative binomial mixed effects models were fitted for MK alpha and dense granule number accounting for the fixed effects ploidy, genotype and their interaction and modelling the fluctuations amongst individuals as random effects. An analogous linear mixed effects model was fitted to the log of MK cell size. The interaction term will capture the ploidy-dependent slope, being the rate of the increase in granule number and cell size.

**Figure 6. Minor intron retention in mature Roifman MK for two candidate genes.**

(A) Sashimi plots showing minor intron regions (in between orange lines) for DIAPH1 (intron 11-12) and HPS1 (intron 16-17). Minor intron retention was identified via RNAseq analsysis of mature MK from Roifman patients P1 (red) and P2 (green), while these introns were not retained in MK of healthy controls (black). (B) RT-PCR analysis on RNA of mature MK was performed to confirm minor intron retention in DIAPH1 and HPS1 for all three Roifman patients.

**Figure 7. Cytoskeletal aberrations in platelets of Roifman patients.**

Representative immunostaining of poly-L-lysine immobilized platelets for cytoskeletal components (A) alpha tubulin and (B) F-actin. Corrected total cell fluorescence (CTCF) values were compared for two healthy controls vs. P1 and P3. In both graphs, box and whiskers represent ranges of 25 to 75% and 5 to 95%. Linear mixed effects models were fitted to the CTCF values of both cytoskeletal components accounting for the genotype as fixed effect and the different individuals as random effects. (C) DIAPH1 protein levels were evaluated by Western blot in parallel with platelet marker integrin β3 and GAPDH (two healthy controls vs. P1-2-3).