Loss of B cells and their precursors is the most constant feature of GATA-2 deficiency in childhood myelodysplastic syndrome

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Online Supplementary Appendix

Summary of clinical findings in *GATA2* non mutated patients

Clinical findings of GATA-2-deficient cases are summarized in table 2. We carefully reviewed clinical data of remaining patients during the study. Generally pediatric patients with primary MDS are suspect of having primary germline aberrancy associated with bone marrow failure/MDS. Among GATA-2-deficient patients 7 out of 12 were suspect of having primary genetic cause based on abnormalities outside hematopoietic system or clinical behavior (table 2).

Among patients with advanced MDS *GATA2* wildtype we found 4 out of 20 patients with suspect germline aberrancy:

pt1: family history – father with thrombocytopenia, very young child – first symptoms already at age of 6 months, manifestation of advanced MDS at age of 10 months, stigmatization

pt2: skin lesions present at 6 months, xanthogranuloma at age of 1 year, MDS manifested at age of 4 years

pt3: young child diagnosed at age of 1 year, complex karyotype, manifested with unexpectedly severe acute GVHD after stem cell transplantation

pt4: urogenital abnormalities

Among RCC patients we found 5 patients out of 27 patients with suspect germline abnormality:

pt1: immunodeficiency

pt2: family history (mother after SCT for MDS RAEB)

pt3: stigmatization, nail dystrophy

pt4: cardiac abnormalities, stigmatization

pt5: suspect family history (cousin suffered from ALL)

No AA patient was suspect to have primary genetic abnormality based on reviewing of clinical data.

Diagnostic criteria

RCC is a provisional entity that is characterized by persistent cytopenia with dysplasia and <5% blasts in BM. All patients with suspected MDS or AA underwent trephine biopsy, and two pathologists (VC or GK) performed all analyses. The distinction between RCC and AA was based on the presence or absence of patchy left-shifted erythropoiesis as the primary differentiating parameter[1]. Patients with cytogenetic aberration were classified as MDS even if the dysplasia in histopathology was absent. The "Non-RCC" group consisted of patients with advanced forms of MDS (RAEB, RAEB-t and

myelodysplasia related (MDR)-AML). Classification of advanced forms of MDS was performed according Hasle et al.[2] and the 2008 WHO classification[3] as follows. RAEB was defined by the presence of 2-19% of blasts in PB and/or 5-19% of blasts in BM; RAEB-t had 20-30% of blasts in PB or BM and AML1/ETO and CBFb/MYH11 fusion genes were absent. MDR-AML was defined by MDS related cytogenetic abnormality or when multilineage dysplasia was morphologically evident and more than 30% of blasts was present by morphology.

Flow cytometry and definition of subpopulations

BM and PB samples were collected in EDTA-containing tubes and analyzed within 24 hours. Sample cellularity was assessed using flow cytometry via calculation from the sample flow rate (5 µl of sample diluted in 250 µl of ammonium chloride solution). Selected sample amounts corresponded to 100,000-200,000 acquired events per tube. Sample preparation consisted of a 15-min incubation with monoclonal antibodies (mAb) used at the sample-to-mAb volume ratios recommended by the manufacturers. Red blood cells were lysed during a 15-min incubation in ammonium chloride, followed by a 5-min centrifugation (500 g). The supernatant was discarded, phosphate-buffered saline (PBS) was added, and data acquisition was immediately begun. Cells were acquired using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA), DAKO Cyan (Dako, Glostrup, Denmark) or BD LSR II (BD Biosciences, San Jose, CA, USA), and the data were analyzed using FlowJo software (TreeStar, Ashland, Oregon, USA). The mAb combinations used in the study are described in Supplementary table 1. All populations were estimated as a percentage of nucleated cells defined using SYTO-16 or SYTO-41 nucleated dye (Thermo Fisher Scientific Inc., Waltham, MA, USA). The following populations were analyzed: monocytes ($CD14^{pos}45^{pos}SSC^{med}$); granulocytes ($CD45^{pos}SSC^{high}$); lymphocytes ($CD45^{pos}SSC^{low}$); red cells ($CD45^{neg}71^{bright}SSC^{low}$); $CD34^{pos}$ and $CD117^{pos}$ precursors; B cells ($CD19^{pos}$); T cells ($CD3^{pos}$); and NK cells (CD16 or $CD56^{pos}3^{neg}$). Supplemental table 1 describes the detailed composition of the panel. Analysis of monocyte count in peripheral blood was performed by hematological analyzer and/or microscope.

Cytogenetics

Cytogenetic and FISH analyses were performed prior to therapy according to standard protocols. BM cells were cultivated for 24h in RPMI 1640 medium with 10% fetal calf serum without mitogenic stimulation. Chromosomal preparations were made according to conventional techniques using colcemide, hypotonic treatment, fixation in methanol/acetic acid and Wright stain (G-banding). The karyotypes were characterized according to the International System of Human Cytogenetic Nomenclature. All patients were screened for monosomy 7 or deletion 7q and trisomy 8, using interphase fluorescence in-situ hybridization (I-FISH). All FISH assays were performed according to the manufacturer´s recommendations.

DNA isolation

DNA was isolated from 200 µl of fresh PB and BM or frozen cell pellets using a QIAamp DNA Blood Mini Kit or QIAamp DNA Blood Micro Kit (Qiagen GmbH, Hilden, Germany). A retrospective search for dried newborn blood spots (DBS, Guthrie cards) was performed after obtaining written informed consent from patients or their guardians in 4 *GATA2*-mutated patients (UPN1, UPN6, UPN8, and UPN11).

A circle with a diameter of 3.2 µm was cut from each DBS, and DNA was eluted at 99°C for 1 hour in a shaker (500 rpm) using 100 µl of Generation DNA Elution Solution (Qiagen GmbH, Hilden, Germany) supplemented with 100 µg/ml of yeast tRNA (Life Technologies, Carlsbad, CA, USA)[4].

GATA2 sequencing

GATA2 mutation status was investigated in all MDS/AA patients with available material. Genomic DNA was extracted from BM or PB samples. The entire coding region of *GATA2* and an intronic enhancer region 3' to exon 6 were amplified using genomic PCR and directly sequenced using primers specified in Supplementary table 2. Sequences were visualized, aligned to the reference sequence and analyzed using BioEdit software[5]. Available parents of all *GATA2*-mutated patients who agreed to the analysis were investigated to assess the familiar origin of the particular *GATA2* mutations.

Statistics

Statistical analyses were performed using Statistica (StatSoft, Inc., Tulsa, OK, USA) and GraphPad (GraphPad Software, Inc., La Jolla, CA, USA). The results were considered significant when p values were less than 0.05.

1. Baumann I, Führer M, Behrendt S, et al. Morphological differentiation of severe aplastic anaemia from hypocellular refractory cytopenia of childhood: reproducibility of histopathological diagnostic criteria. Histopathology. 2012;61(1):10–17.
2. Hasle H, Niemeyer CM, Chessells JM, et al. A pediatric approach to the WHO classification of myelodysplastic and myeloproliferative diseases. Leuk Off J Leuk Soc Am Leuk Res Fund UK. 2003;17(2):277–282.
3. Baumann I, Niemeyer CM, Bennett JM, Shannon K. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al., editors. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues. Lyon: Internatioal Agency fo Research on Cancer (IARC); 2008. page 104–107.
4. Froňková E, Klocperk A, Svatoň M, et al. The TREC/KREC assay for the diagnosis and monitoring of patients with DiGeorge syndrome. PLoS One. 2014;9(12):e114514.
5. Hall TA. Pesticides: Managing Risks and Optimizing Benefits. Ragsdale NN, Seiber JN, editors. Nucleic Acids Symposium Series. Washington, DC: American Chemical Society; 1999. 95-98 p.

Supplementary figures and tables

Supplementary figure 1.

Flow chart describing number of analyzed patients. Three patients assigned as “others” are GATA-2-deficient patients followed for immunodeficiency and changes in blood count (UNP2, UPN7 and UPN8). Two of these patients developed MDS in early adulthood (UPN2 and UPN7).

Supplementary figure 2.

Correlation of age and levels of assessed cell types in bone marrow and KREC and TREC in both BM and PB in patient groups and in controls.

Statistical dependence of age and each parameter was tested using the Spearman rank correlation coefficient. The R in the graph indicates a correlation coefficient in samples, which is shown only where p values are lower than 0.05. No significant correlation of age and respective parameter was found in GATA-2-deficient patients. The dots represent controls, and the larger shapes represent patient categories: triangle, GATA-2; square, RCC; rhombus, non RCC; inversed triangle, AA. The gray lines represent semi-log trends and are shown only when the Spearman rank correlation p value is lower than 0.05.

Supplementary figure 3.

Consecutive samples of UPN9 are shown in dot plots CD117 against side scatter (Ssc). In lower line B cell phenotype of blasts is shown by CD10 and CD20 expression. Graph shows percentage of different phenotype of blasts in bone marrow in consecutive samples.

Supplementary figure 4.

Absolute and relative monocyte counts in PB are shown in follow up samples of GATA-2-deficient patients. Grey lines represent normal values. Day 0 represents day of hematopoietic transplantation or death or last follow up.

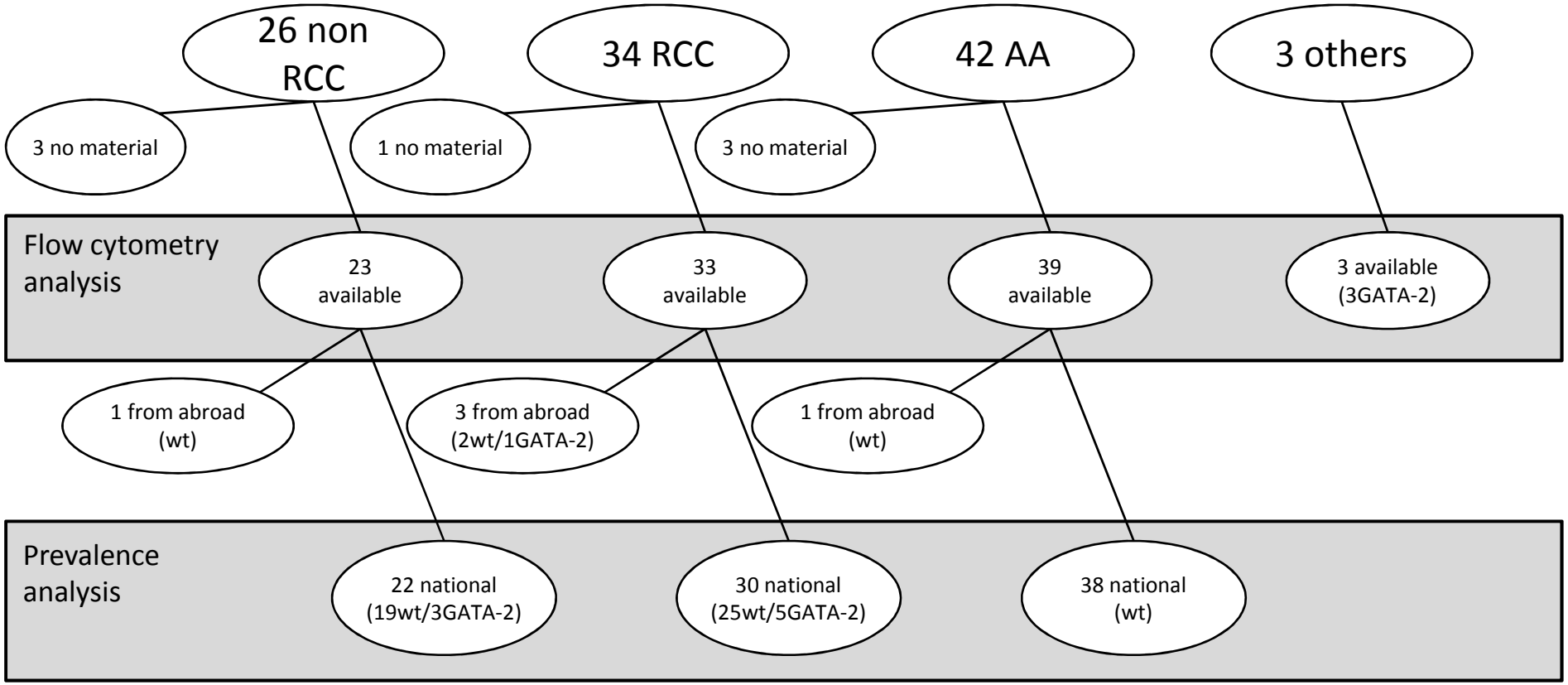
Supplementary table 1.

Antibody panels and antibody clones used in our study are shown in Supplementary table 1.

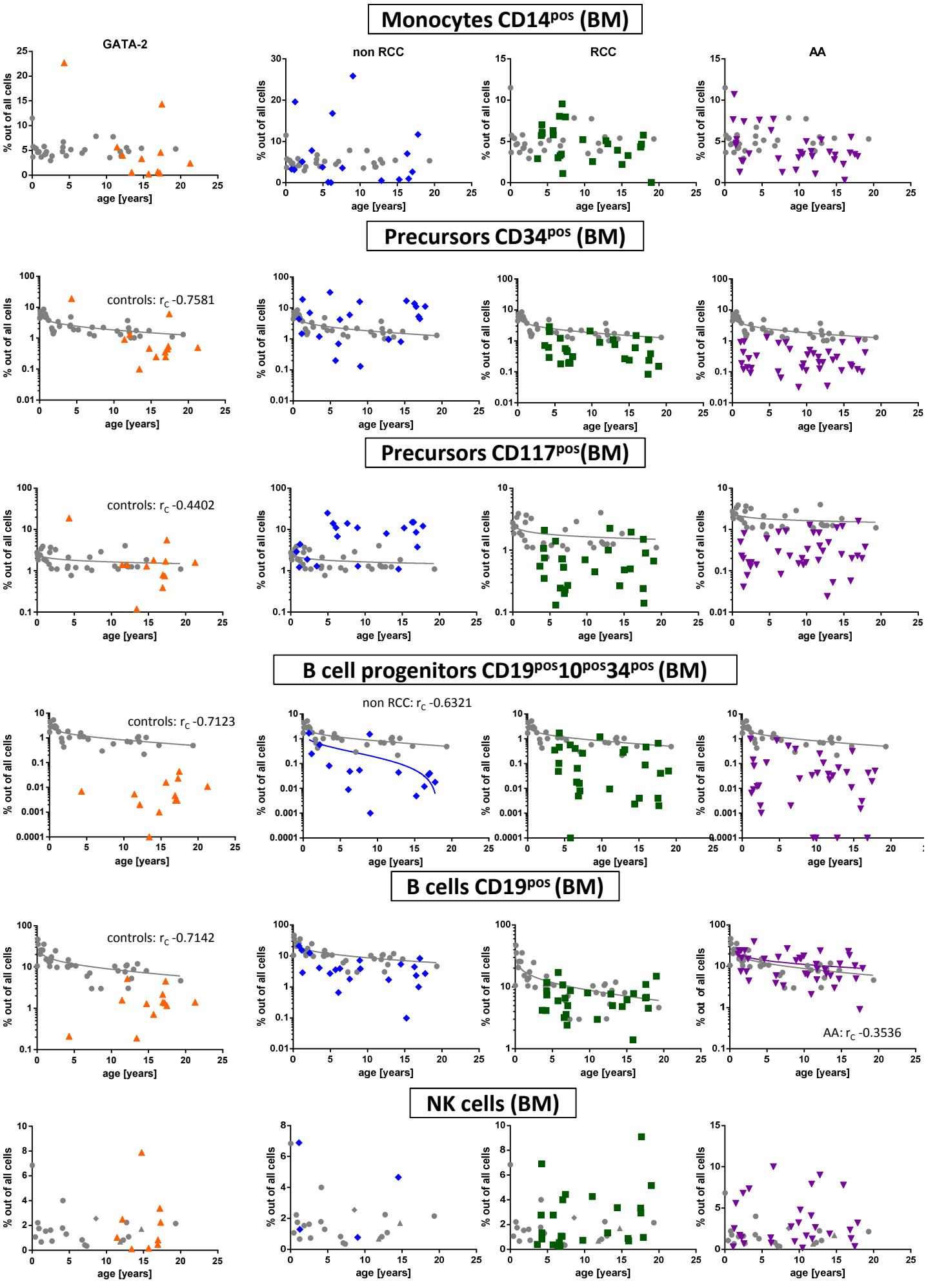
Supplementary table 2.

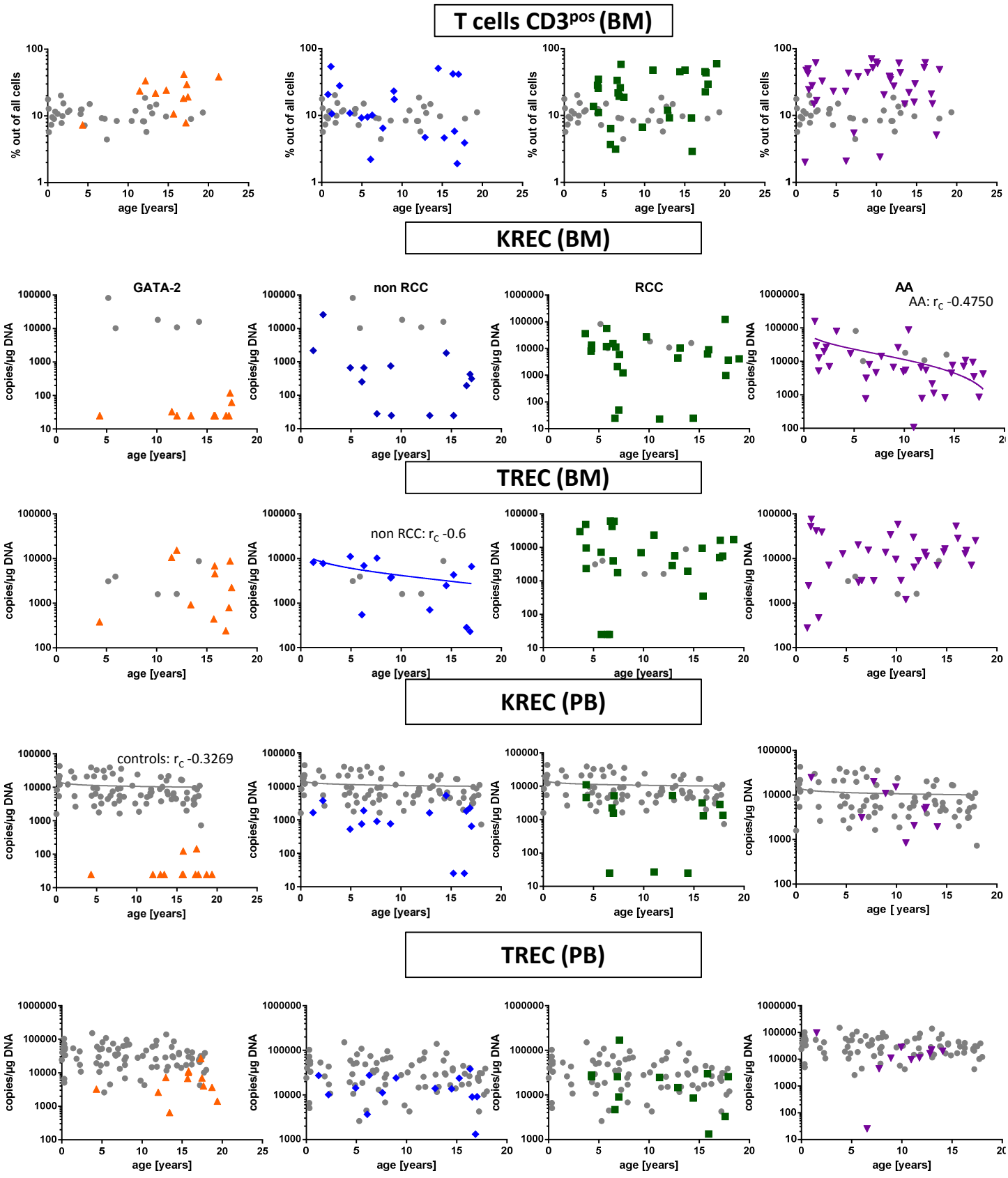
Primer sequences used for PCR and sequencing and genomic coordinates of regions analyzed for mutational status.

Supplementary figure 1



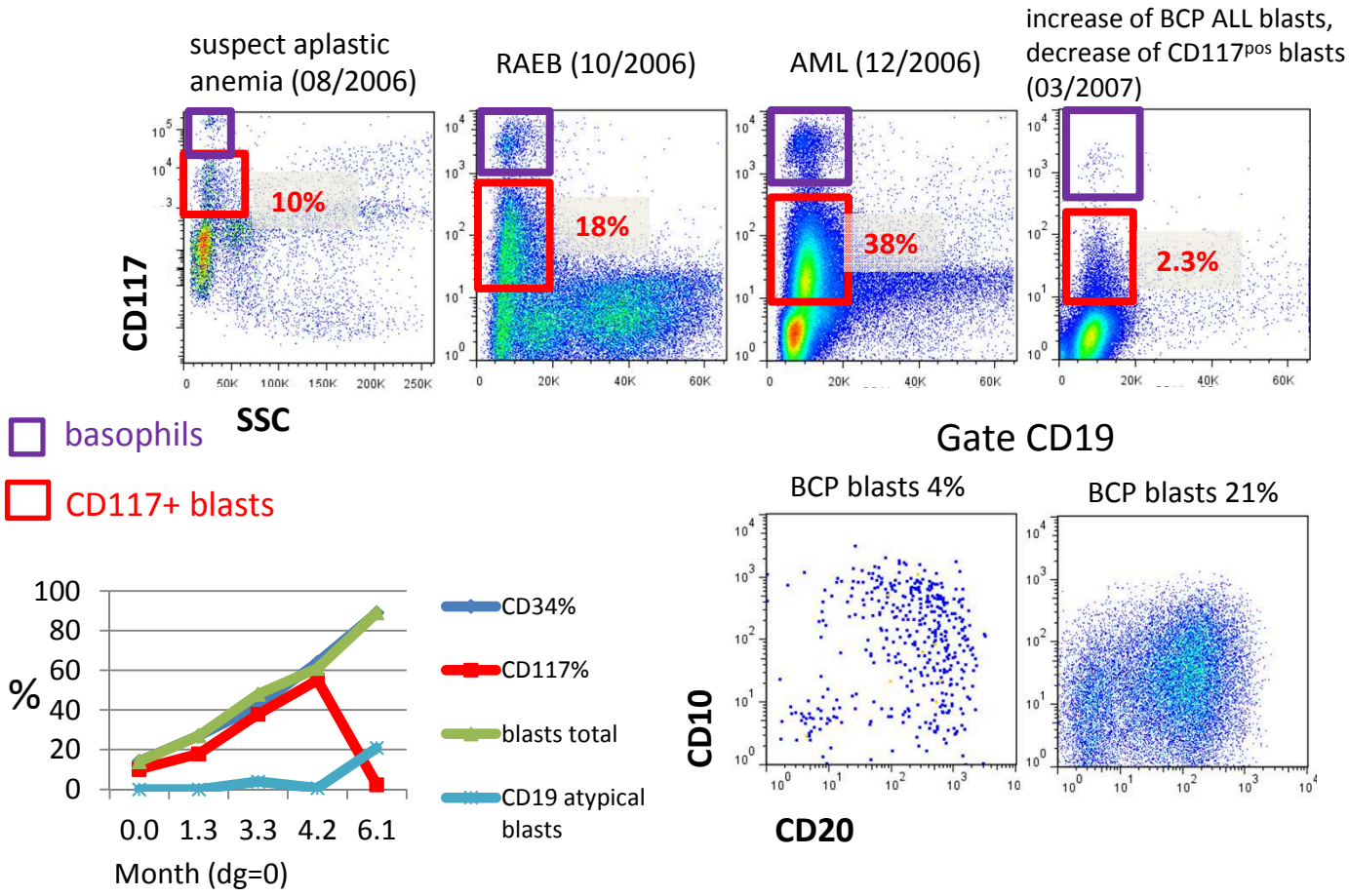
Supplementary figure 2




T cells CD3pos (BM)
% out of all cells
age [years]
KREC (BM)
GATA-2
non RCC
RCC
AA
AA: rc -0.4750
copies/µg DNA
TREC (BM)
non RCC: rc -0.6
KREC (PB)
controls: rc -0.3269
TREC (PB)

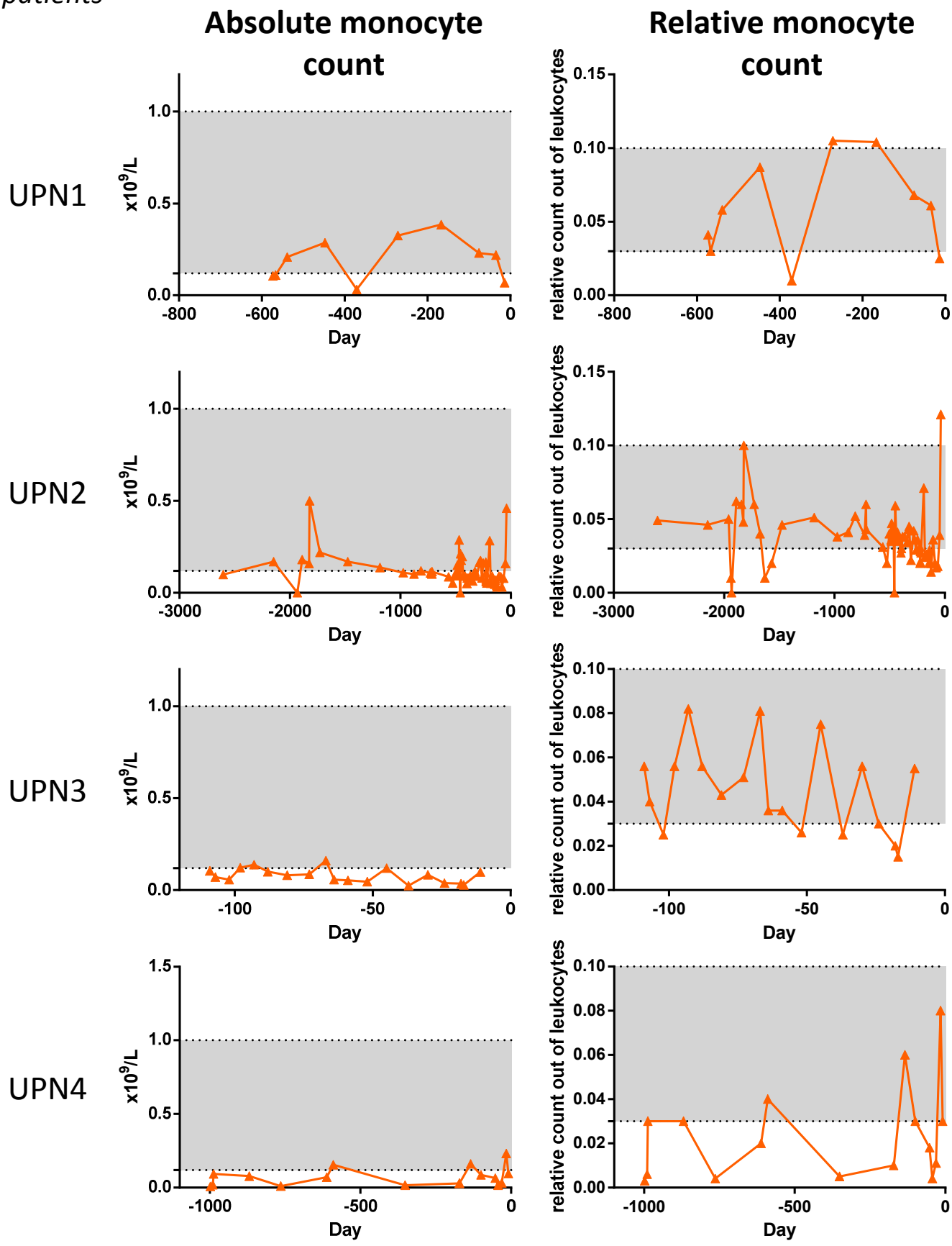
Supplementary figure 3

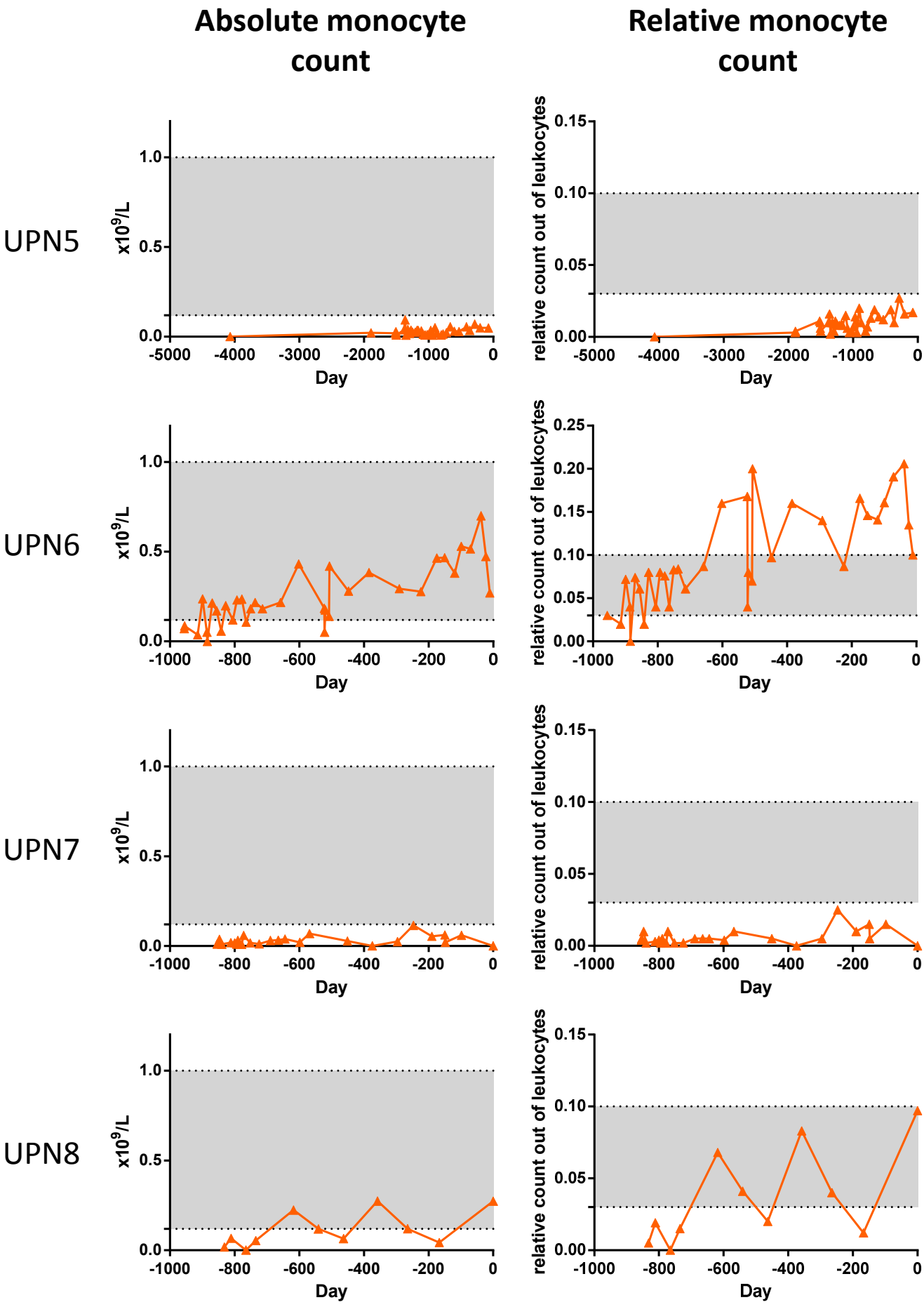
Development of B cell phenotype in blasts in GATA2-mutated patient. (UPN9)

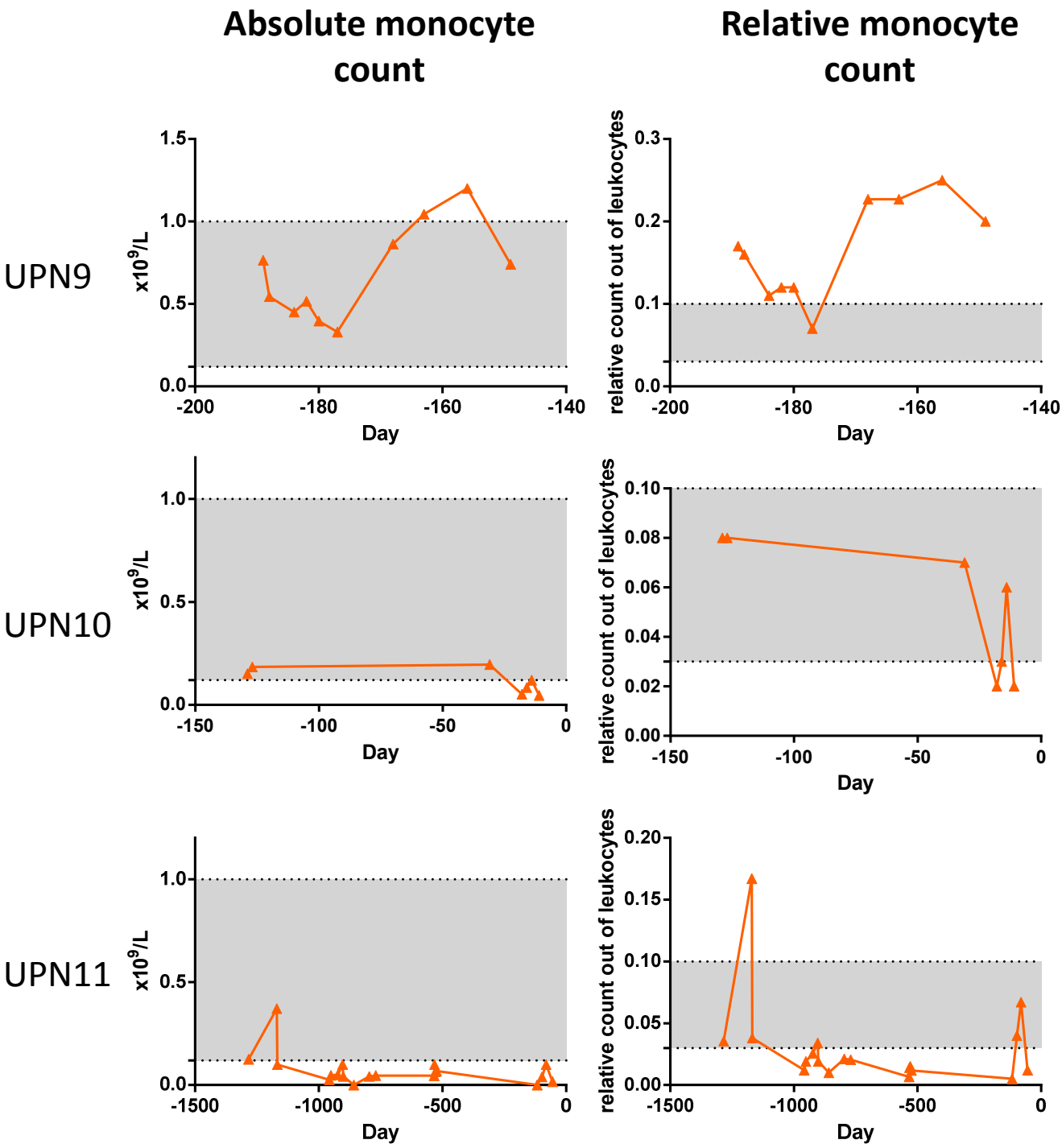


Supplementary figure 4

Relative and absolute monocyte counts during the follow up of GATA-2-deficient patients




Absolute monocyte count
Relative monocyte count
UPN5
UPN6
UPN7
UPN8
x10^9/L
relative count out of leukocytes
Day
1.0
0.5
0.0
0.15
0.10
0.05
0.00
0.25
0.20
-5000
-4000
-3000
-2000
-1000
-800
-600
-400
-200
0


Absolute monocyte count
Relative monocyte count
UPN9
UPN10
UPN11
x10^9/L
relative count out of leukocytes
Day
1.5
1.0
0.5
0.0
0.3
0.2
0.1
-200
-180
-160
-140
0.10
0.08
0.06
0.04
0.02
0.00
-150
-100
-50
0
0.20
0.15
0.10
0.05
-1500
-1000
-500

Supplementary table 1. Antibody panel and used antibody clones

	FITC/Syto 16	PE	PE-Texas Red	PerCP	PE-Cy7	APC	Alexa 700	Syto 41
Tube 1	HLA-DR	CD14	CD4	CD45	CD3	CD8		Syto 41
Tube 2	CD99	CD7		CD45	CD5	CD3		
Tube 3	CD20	CD10		CD45	CD19	CD34	CD38	Syto 41
Tube 4	CD15	CD117	CD7	CD45	CD34	CD33		
Tube 5	CD3	CD16/56		CD45		CD19		
Tube 6	SYTO16	CD117		CD45	CD34	CD33	CD71	
Tube 7	SYTO16	GPA		CD45	CD117	CD34	CD71	

Antigen	Fluorochrome	Clone	Manufacturer
CD3	FITC	SK7	Becton Dickinson
CD3	APC	SK7	Becton Dickinson
CD3	PE-Cy7	UCHT1	Beckman Coulter
CD4	ECD	SFCI12T4D11	Beckman Coulter
CD5	PE-Cy7	BL1a	Beckman Coulter
CD7	PE-Texas Red	8H8.1	Beckman Coulter
CD7	PE	8H8.1	Beckman Coulter
CD8	APC	MEM-31	Exbio
CD10	PE	SS2/36	DAKO
CD14	PE	MEM-15	Exbio
CD15	FITC	MMA	Becton Dickinson
CD16	PE	B73.1	Becton Dickinson
CD19	PE-Cy7	J3119	Beckman Coulter
CD19	APC	SJ25C1	Becton Dickinson
CD20	FITC	L27	Becton Dickinson
CD33	APC	D3HL60.251	Beckman Coulter
CD34	APC	581	Beckman Coulter
CD34	PE-Cy7	581	Beckman Coulter
CD38	Alexa 700	HIT2	Exbio
CD45	PerCP	2D1	Becton Dickinson
CD56	PE	NCAM 16.2	Becton Dickinson
CD71	Alexa 700	MEM-75	Exbio
CD99	FITC	TÜ12	Becton Dickinson
CD117	PE	95C3	Beckman Coulter
CD117	PE-Cy7	104D2D1	Beckman Coulter
GPA	PE	11E4B-7-6 (KC16)	Beckman Coulter
HLA-DR	FITC	Immu-357	Beckman Coulter
Syto 41	PE		Life Technologies
Syto16	PE-Cy7		Life Technologies

Supplementary table 2. Primer sequences used for PCR and sequencing and genomic coordinates of regions analysed for mutational status

exon[&]	primer type	primer sequence	PCR amplicon length (bp)	genomic coordinates of analyzed regions *
4	PCR forward, sequencing	GGACTGGTGCTCTTTCTCGCC	508bp	chr3:128205646-128205000
	PCR reverse, sequencing	CAGCTCGATTCCTGCGGATC		
5	PCR forward, sequencing	TGATCTTTCTGCCCACCCTGAT	736 bp	chr3:128204570-128205000
	PCR reverse, sequencing	AAAAACGCAAATGCTCCCCTCTT		
	sequencing	CGTGAGCCCCTTCTCCAAGAC		
	sequencing	GCGTCTCCAGCCTCATCTTCC		
	sequencing	GGAAGATGAGGCTGGAGACGC		
6	PCR forward, sequencing	GTTAAGCAGGCCCCCGTGT	800 bp	chr3:128202131-128202000
	PCR reverse, sequencing	AGGCAGGACTGAGCTGAGGAGAC		
	sequencing	CCTGTAATTAACCGCCAGCTCCT		
	sequencing	GCCAGATTTCCTCCTCGGG		
7	PCR forward, sequencing	CCTGCTGACGCTGCCTTG	253 bp	chr3:128200662-128200000
	PCR reverse, sequencing	CAAGCCAAGCTGGATATTGTG		
8	PCR forward, sequencing	AGGAAGGAACTGGCCCTCTGA	365 bp	chr3:128199862-128200000
	PCR reverse, sequencing	CGGTCCTCGACGTCCATCTGTT		

[&] exons are numbered according to NG029334.1 NCBI Reference sequence

* genomic coordinates areaccording to GRCh37/hg19; analysed regions include coding sequences of individual exons and 5' part of inron 6 adjacent to exon 6