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ORIGINAL PAPER

Evolution of severe (transfusion-dependent) anaemia in myelodysplastic syndromes with 5q deletion is characterized by a macrophage-associated failure of the eythropoietic niche

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Summary

Evolution of erythrocyte transfusion-dependent (RBC-TD) anaemia associated with haploinsufficiency of the ribosomal protein subunit S14 gene (*RPS14*) is a characteristic complication of myelodysplastic syndromes (MDS) with del(5q) [MDS.del(5q)]. Evaluating 39 patients with MDS.del(5q), <5% of anaemia progression was attributable to *RPS14*-dependent alterations of normoblasts, pro-erythroblasts, or $CD34^+CD71^+$ precursors. Ninety-three percent of anaemia progression and 70% of the absolute decline in peripheral blood Hb value were attributable to disappearance of erythroblastic islands (Ery-Is). Ery-Is loss occurred independently of blast excess, *TP53* mutation, additional chromosome aberrations and *RPS14*-dependent alterations of normoblasts and pro-erythroblasts. It was associated with *RPS14*-dependent intrinsic ($S100A8^+$) and extrinsic [tumour necrosis factor α (TNF-α)-overproduction] alterations of ($CD169^+$) marrow macrophages ($p < 0.00005$). In a mouse model of *RPS14* haploinsufficiency, Ery-Is disappeared to a similar degree: approximately 70% of Ery-Is loss was related to *RPS14*-dependent S100A8

Guntram Buesche and Huesniye Teoman contributed equally to this work.

In honour of Prof. Dr. med. Axel Georgii (2 August 1927–7 February 2021), founder, first Chair and Director of the Institute of Pathology, Hannover Medical School, Germany.

overexpression of marrow macrophages, less than 20% to that of CD71^{high}Ter119^{-} immature precursors, and less than 5% to S100A8/p53 overexpression of normoblasts or pro-erythroblasts. Marked Ery-Is loss predicted reduced efficacy (erythrocyte transfusion independence) of lenalidomide therapy (p = 0.0006). Thus, erythroid hypoplasia, a characteristic complication of MDS.del(5q), seems to result primarily from a macrophage-associated failure of the erythropoietic niche markedly reducing the productive capacity of erythropoiesis as the leading factor in anaemia progression and evolution of RBC-TD in MDS.del(5q).

KEYWORDS
5q deletion, erythroblastic island, erythropoiesis, myelodysplastic syndromes, *RPS14*

INTRODUCTION

Anaemia, the leading symptom of myelodysplastic syndromes (MDS), results from alterations of erythropoiesis, such as increased apoptosis, dysplastic features, and impaired maturation of nucleated erythroid cells (NEC).[1–4] In 10%–20% of MDS patients, the background of anaemia is haploinsufficiency of a gene located on the long arm of chromosome 5 [del(5q)], the most frequent chromosomal aberration in MDS,[5] encoding for the ribosomal protein subunit S14 (*RPS14*).[6] Impaired ribosome formation due to reduced S14 protein synthesis induces MDM2 protein degradation, consecutive p53 protein overexpression, increased expression of proteins involved in innate immune signalling, the heterodimeric S100A8/S100A9 proteins, resulting in a deficiency of normoblasts.[7,8] The relevance of reduced *RPS14* expression was confirmed by experiments rescuing defective erythropoiesis by RPS14 upregulation, normalization of p53 protein expression in cells with del(5q)[9–11] and genetic inactivation of S100A8 expression in *RPS14*-haploinsufficient haematopoietic stem cells.[12] These observations link anaemia in MDS with del(5q) [MDS.del(5q)] to congenital ribosomopathies.[13] Two features are unclear up to now:

1. A great variation in the degree of anaemia among patients with the same karyotype aberration, especially the same common deleted region (CDR).
2. The background of anaemia progression resulting in erythrocyte (RBC) transfusion dependence (RBC-TD) in almost all patients with MDS.del(5q).[14]

Severe anaemia and RBC-TD indicate more advanced stages of MDS, independently predicting increased risk of secondary acute myeloid leukaemia (sAML) and shortened survival time of patients.[15,16] A common assumption is that marked deterioration of anaemia results from progression of MDS, such as blast excess, clonal evolution, sAML, marrow hypoplasia or fibrosis (MF),[3,17] occurrence of additional chromosome aberrations[5] or unfavourable mutations.[17–19] However, a significant proportion of MDS.del(5q) patients suffers from anaemia aggravation without or prior to the evolution of these features.[4,14] It is unclear until now whether *RPS14*-dependent deficiency of normoblasts is the only background of this aggravation. Alterations of erythroblastic islands (Ery-Is),[20] which provide the erythropoietic niche, are not regarded in current concepts of dyserythropoiesis in MDS.del(5q),[2] although they are essential to RBC production[21] and seem to contribute to anaemia and prognosis in MDS patients.[4] Therefore, we evaluated alterations of erythropoiesis associated with anaemia progression during the natural course of MDS.del(5q) [during best supportive care (BSC) or during transient ineffective therapy] considering factors which might be relevant to anaemia progression or to alterations of Ery-Is, such as MDS.del(5q) subtype, risk score and indicators of progressive disease. The results were compared with those in normal (healthy) bone marrow (BM) and in an animal model of *RPS14*-haploinsufficiency.

MATERIALS AND METHODS

Patients

We restricted our study to a well-defined group of 39 patients:

1. With MDS.del(5q) and less than 10% blasts in diagnostic blood and BM samples and International Prognostic Scoring System (IPSS)-intermediate-1 or -low risk[22];
2. whose disease was diagnosed prior to approval of lenalidomide therapy of lower-risk MDS.del(5q) in Europe;
3. who had been treated by BSC (or a transient ineffective therapy) from the day of diagnosis of MDS.del(5q) until the inclusion in the MDS-003 study (ClinicalTrials.gov number, NCT00065156) [23–25]; and
4. whose course was monitored by BM biopsies (BMBs), BM aspirates (BMAs), and cytogenetic BM analyses at regular six- to 12-month intervals after diagnosis of disease.

Within a median period of 34 months with BSC or a transient ineffective therapy (mean ± SD: 38 ± 33 months), RBC-TD occurred in all patients. Nineteen received all-trans retinoic acid (ATRA) ± tocopheral-α and six erythropoietin

TABLE 1 Characteristics on the day of diagnosis and at study entry: seven patients showed one additional chromosome aberration besides del(5q), three a clonal evolution, none a complex karyotype or a very high IPSS-R score

Means ± standard deviation or ratios (%)	MDS.del(5q) [*n* = 39]		Control group [*n* = 33] with normal marrow
	On the day of diagnosis	At MDS-003 study entry	
Age [years]	64.2 ± 11.5	67.2 ± 11.2	66.4 ± 10.7
Sex [male]	35.9%	35.9%	42.2%
Haemoglobin [g/l]	93 ± 15	94 ± 15	138 ± 19
Prior to transfusions	9.3 ± 1.5	7.8 ± 1.3	
Transfusion dependency	41.0%	100.0%	0.0%
Neutrophils [10^9/l]	2.3 ± 1.3	2.2 ± 1.2	4.3 ± 2.3
Platelets [10^9/l]	324 ± 256	245 ± 168	279 ± 115
Previous ATRA + tocopherol-α, erythropoietin	0.0%	48.7%	0.0%
Karyotype			
Isolated del(5q)	79.5%	73.5%	0.0%
+ one further aberration:	17.9%	25.5%	0.0%
+21	5.1%	7.7%	
inv(9)	5.1%	5.1%	
–Y, –7, inv(5)[a]	7.7%	7.7%	
t(1;3), inv(3)[a]	0.0%	5.1%	
Mutations			
TP53	17.9%	17.9%	0.0%
JAK2 (V617F)	9.1%	9.1%	
MPL, *CALR*	0.0%	0.0%	
WHO classification			
MDS-MLD	0.0%	2.0%	
Isolated del(5q)	92.3%	71.4%	
MDS-EB-1	7.7%	26.5%	
Further features			
MF	7.7%	10.3%	0.0%
Ring sideroblasts >15%	15.4%	15.4%	0.0%
IPSS-R			
Very low risk		7.7%	
Low risk		64.1%	
Intermediate risk		20.5%	
High risk		7.7%	
Erythropoiesis			
Log($CD34^+CD71^+$/mm^3)	1.23 ± 0.76	0.89 ± 0.97	−0.01 ± 1.07
Log(Ery-Is/mm^3)	3.85 ± 1.20	3.05 ± 2.15	5.97 ± 0.95
Log(HbA^-CD71^+ NEC)[b]	1.70 ± 1.81	1.67 ± 1.75	2.20 ± 0.78
Log(HbA^+ NEC)[b]	2.31 ± 1.40	1.75 ± 1.58	2.43 ± 0.72
Gene expression			
RPS14 mRNA [−ΔΔCT][c]	−1.29 ± 1.88	−1.77 ± 2.16	−0.17 ± 0.91
TNF-α mRNA [−ΔΔCT][c]	2.06 ± 2.34	2.68 ± 1.83	−0.03 ± 0.21

Note: Three patients showed a *JAK2* mutation without proliferative features. Since the cell counts followed a log-linear or a Poisson distribution and the calculations were performed applying log-linear and Poisson regression models, the means of logarithmic values of the numerical densities of erythropoietic components were presented.

Abbreviations: ATRA, all-trans retinoic acid; *CALR*, calreticulin; Ery-is, erythroblastic islands; IPSS-R, Revised International Prognostic Scoring System; MDS, myelodysplastic syndrome; MF, marrow fibrosis; MLD, multilineage dysplasia; *MPL*, myeloproliferative leukaemia protein; NEC, nucleated erythroid cell; *RPS14*, ribosomal protein subunit S14; *TNF*-α, tumour necrosis factor α.

[a]Three patients showed –Y, –7 or inv(5), two other *t*(1;3) or inv(3) besides del(5q).

[b]Counts per Ery-Is section.

[c]Relative to the median CT value of the control group with normal marrow.

for 3–6 months neither with a relevant increase (≥20 g/l) in blood Hb value nor with reversal of RBC-TD. The median period of follow-up after entering the MDS-003 study was 45 months.

Thirty-three age- and sex-matched persons with healthy BM neither showing (a history of) malignant neoplasa, nor inflammatory disease, nor a history of radiation or chemotherapy served as controls. The characteristics of both groups are presented in Table 1 and Table S1. All analyses were conducted in accordance with the Declaration of Helsinki and the rules of the Ethics Committee of Hannover Medical School.

Degree and progression of anaemia

The degree of anaemia was defined as the squared deviation of the Hb value(s) of peripheral blood from those of the controls. Analyses of progression, aggravation or exacerbation of anaemia focussed on the (time-dependent) decline in Hb values prior to RBC transfusion(s).

Morphologic evaluation

Diagnosis and classification of disease were performed according to WHO criteria.[26] Erythropoiesis was evaluated for atypia(s) in more than 10% of NEC, impaired maturation, apoptosis above 1% of NEC, Ery-Is, and nuclear p53 protein overexpression. Details on alterations of erythropoiesis other than those of Ery-Is were not presented, but the sum of their effects on the degree of anaemia was. Ery-Is and NEC were determined by a novel exact approach published by our

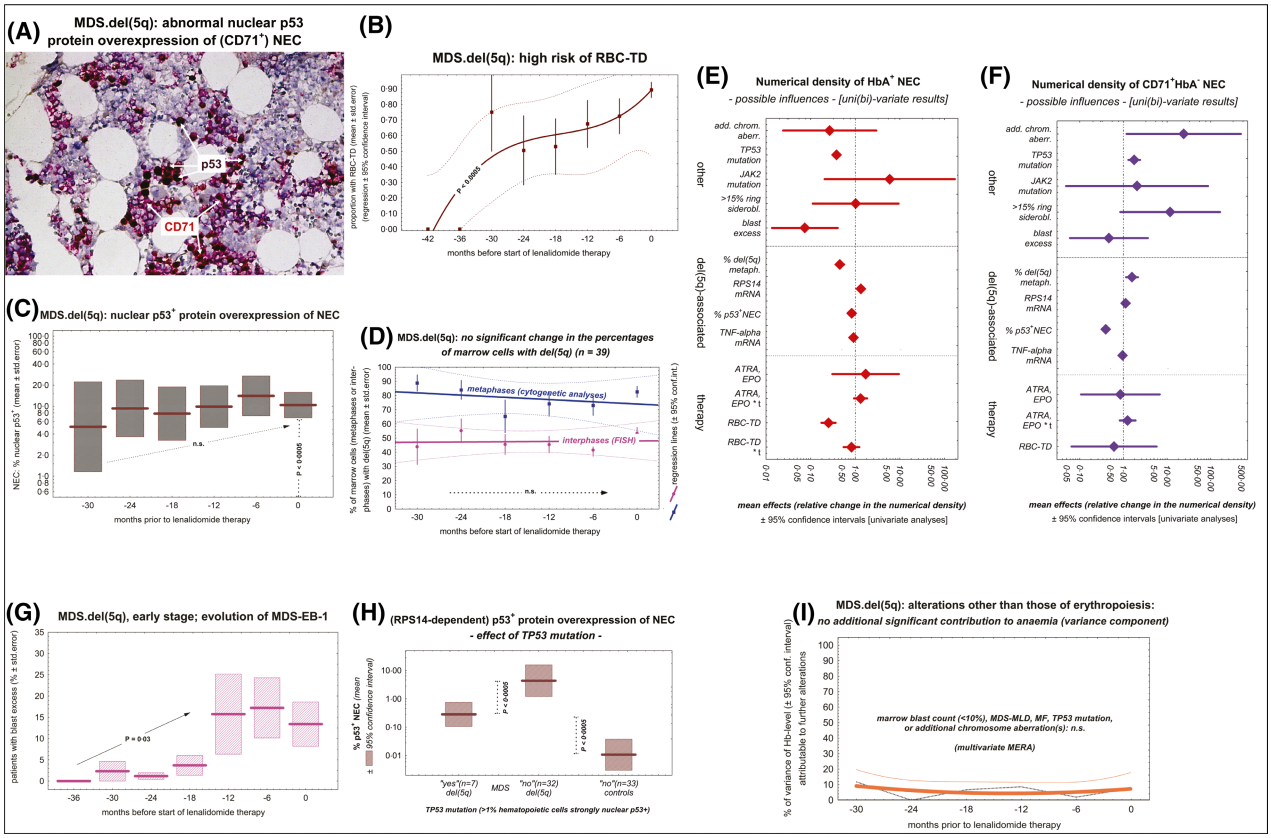


FIGURE 1 Anaemia and its progression in MDS.del(5q): In MDS.del(5q) (±other chromosome aberrations), anaemia was associated with abnormal nuclear p53 protein overexpression of nucleated erythroid cells (NEC; A): anti-CD71/anti-p53 double staining, magnification 250×. Within three years of follow-up, the rate of severe erythrocyte transfusion-dependent (RBC-TD) anaemia progressed towards >90% (B). This progress did not relate to an aggravation of the [ribosomal protein subunit S14- (RPS14–)/p53-dependent] alterations at the level of normoblasts and pro-erythroblasts (C), nor to changes in the percentages of cells with del(5q) (D), nor to occurrence of additional chromosome aberrations, *JAK2* mutation, ring sideroblasts, blast excess, nor to a transient therapy with all-trans retinoic acid (ATRA) + tocopherol-α or erythropoietin (E–G; **t* = adjusted to the period of time since diagnosis of MDS). The narrowness of the confidence intervals of the effects of the variables associated with del(5q) indicate their signifcance to the numerical densities of NEC (E, F). *TP53* mutation (determined by >1% marrow cells with strong nuclear p53 positivity) was associated with lower percentages, but not a complete loss of NEC with abnormal nuclear p53 protein overexpression (H). Neither blast excess <10% of marrow cells (G), nor *TP53* mutation, nor karyotype aberrations other than del(5q) independently contributed to aggravation of anaemia [I; multivariate mixed-effects (Poisson) regression analysis (MERA)]

group a few years ago[4] (1) performing immunophenotyping (anti-CD34, anti-CD71, anti-HbA, anti-p53, anti-TNF-α, anti-CD169, anti-CD68 — including double-stainings) and (2) applying a randomly-positioned clusters model as described previously,[4] using the fact that NEC proliferate and differentiate within clusters.[27] Immature erythroid precursors were determined by $CD34^+CD71^+$ co-expressing cells. MDS with isolated del(5q), no trilineage dysplasia, no blast excess, and no MF were termed 'early phase of MDS.del(5q)'.

Genetic evaluations

Cytogenetic analyses were performed according to standard procedures following the recommendations of the International System for Cytogenetic Nomenclature. The percentage of interphases with del(5q) was determined by fluorescence in situ hybridization (FISH) evaluating at least 200 interphase nuclei.

Gene expression analyses were performed using TaqMan assays for *RPS14*, tumour necrosis factor α (*TNF-α*), and glycophorin A (*GPA*) evaluating all BM cells. *TNF-α* gene expression results were related to TNF-α protein expression of cells applying immunohistochemistry.

All patients were checked for *JAK2*, myeloproliferative leukaemia protein (*MPL*) and calreticulin (*CALR*) mutations[28] using next-generation sequencing as described earlier.[29] *TP53* mutation status was determined on the basis of at least 1% marrow cells with strong nuclear p53 protein expression as presented by Saft et al.[18,30]

Animal model

A well-defined mouse model of *RPS14* haploinsufficiency published several years ago[12] was analysed generating nine chimaeric mice ($RPS14^{-/+}$) with induction of *RPS14* exon 2–4 excision by applying poly(I:C) at the age of 6–8 weeks. Six age- and sex-matched $Mx1Cre^+$ mice served as controls. After sacrifice at 18 months, their BM was evaluated for frequency and S100A8 protein expression of NEC and macrophages

TABLE 2 Influences on NEC during BSC of MDS.del(5q) patients who became RBC-TD ($n = 39$): except for age and sex of patients, risk score, >15% ring sideroblasts and a transient therapy with ATRA + tocopherol-α and/or erythropoietin (19 patients), all variables were significant to the numerical densities of NEC in univariate analyses, but in multivariate analyses, only 1/3 of them remained significant

			HbA⁻ NEC (pro-erythroblasts)				HbA⁺ NEC (normoblasts)			
			Univariate MERA		Multivariate MERA		Univariate MERA		Multivariate MERA	
Predicted change β_i in the counts (numerical densities) of NEC			β_i	***p*-value**	β_i	***p* value**	β_i	***p* value**	β_i	***p* value**
General	Age	On the day of diagnosis (years)		n.s.		n.s.		n.s.		n.s.
	Sex	(male)		n.s.		n.s.		n.s.		n.s.
	Risk score	IPSS-R: (very) low risk (versus other)		n.s.		n.s.		n.s.		n.s.
Genetics	Cytogenetics	% metaphases with del(5q)[a]	+0.007	$p < 0.0005$		n.s.	−0.009	$p < 0.00001$		n.s.
		% interphases with del(5q) (FISH)[a]	+0.011	$p < 0.0005$		n.s.	−0.007	$p < 0.00001$		n.s.
		karyotype: additional aberration(s)[a]	+2.264	$p = 0.05$		n.s.		n.s.		n.s.
	TP53 mutation expression	≥1% marrow cells strongly p53⁺	+0.014	$p = 0.0002$		n.s.	−0.043	$p < 0.00001$		n.s.
		RPS14 mRNA [−ΔΔCT][a]	+0.112	$p < 0.00001$	+0.807	$p < 0.00001$	+0.291	$p < 0.00001$		n.s.
		TNF-α mRNA [−ΔΔCT][a]	−0.045	$p < 0.0005$		n.s.	−0.091	$p < 0.00001$		n.s.
		glycophorin A mRNA [−ΔΔCT][a]	−0.145	$p < 0.00001$	−0.677	$p < 0.00001$	+0.269	$p < 0.00001$	+0.564	$p < 0.00001$
Haematopoiesis	Cellularity	% within bone marrow[a]	−1.547	$p < 0.00001$	+13.01	$p < 0.00001$	−1.366	$p < 0.00001$	+4.502	$p < 0.00001$
	Blast excess	≥5% (MDS-EB-1)[a]	+0.500	$p < 0.00001$		n.s.	−2.617	$p = 0.005$		n.s.
	Erythropoiesis	CD34⁺CD71⁺ precursor cells [/mm³][a]	+0.007	$p < 0.00001$		n.s.	+0.003	$p < 0.00001$	−0.015	$p < 0.00001$
		Ery-Is [/mm³][a]	+0.245	$p < 0.00001$	+0.587	$p < 0.00001$	+0.596	$p < 0.00001$		n.s.
		HbA⁻ NEC [per Ery-I][a]					−9.619	$p < 0.00001$		n.s.
		p53⁺ NEC [ratio][a]	−2.052	$p < 0.00001$		n.s.	−1.072	$p < 0.00001$	−0.002	$p = 0.018$
		atypias >10% of NEC[a]		n.s.		n.s.	−3.474	$p < 0.00001$		n.s.
		Ring sideroblasts >15%		n.s.		n.s.		n.s.		n.s.
		Hb value of blood[a]	+0.096	$p < 0.00001$		n.s.	+0.163	$p < 0.00001$		n.s.
	Macrophages	(CD169⁺) iron-positive [/mm³][a]	+0.002	$p < 0.00001$		n.s.	−0.001	$p = 0.023$		n.s.
Other	Length *t* of time therapy	Since diagnosis of disease [years][a]	−0.466	$p < 0.00001$	−0.646	$p = 0.00001$	−0.454	$p < 0.00001$		n.s.
		ATRA + tocopherol-α, erythropoietin		n.s.		n.s.		n.s.		n.s.
		Duration of therapy [years][a]		n.s.		n.s.		n.s.		n.s.
		RBC-TD		n.s.		n.s.	−1.378	$p < 0.00001$		n.s.

Note: β_i = changes in the numerical density of NEC dependent on alterations of the "independent" variables. MF and mutations of genes other than *TP53* were not considered because of too small numbers of patients.

Abbreviations: ATRA, all-trans retinoic acid; Ery-is, erythroblastic islands; FISH, fluorescence *in situ* hybridization; IPSS-R, Revised International Prognostic Scoring System; MDS, myelodysplastic syndrome; MERA, mixed-effects (Poisson) regression analysis; NEC, nucleated erythroid cell; n.s., not significant (i.e. $p > 0.05$); RBC, erythrocyte; *RPS14*, ribosomal protein subunit S14; TD, transfusion dependence; *TNF*-α, tumour necrosis factor α.

[a]Included as time-dependent variables (i.e. variables changing with time).

TABLE 3 Factors influencing a progression of anaemia during the course of MDS.del(5q) with <10% blasts in BM (BSC, $n = 39$, 33 controls): in univariate analyses, almost all variables were significant to anaemia progression

Predicted change Δ_i in Hb value of peripheral blood (prior to RBC transfusions or the decline occurring during RBC-TD)			Univariate analyses (MERA)		Multivariate stepwise MERA		
			Δ_i	*p* value	Δ_i	LLR	*p* value
General	Period *t* of time	[months]	−0.047	$p < 0.000001$		<1%	n.s.
All marrow cells	*TP53* mutation	[≥1% strongly $p53^+$]		n.s.		<1%	n.s.
	Blast cell count	[% of marrow cells]	−0.081	$p < 0.000001$		<1%	n.s.
	del(5q)	[% of metaphases]	−0.057	$p < 0.000001$		<1%	n.s.
	RPS14 mRNA	[−ΔΔCT]	+0.739	$p = 0.001$		<1%	n.s.
	TNF-α mRNA	[−ΔΔCT]	−0.462	$p = 0.003$		<1%	n.s.
	Macrophages	[$CD169^+$, $iron^+/mm^3$]	−0.000	$p = 0.00001$		<1%	n.s.
Dependence on time *t*	*TP53* mutation × time	[≥1% strongly $p53^+$] × *t*	−0.075	$p < 0.000001$		<1%	n.s.
	Blast cell count × time	[% of cells] × *t*	−0.000	$p = 0.00002$		<1%	n.s.
	del(5q) × time	[% of metaphases] × *t*		n.s.		<1%	n.s.
	RPS14 mRNA × time	[−ΔΔCT] × *t*		n.s.		<1%	n.s.
	TNF-α mRNA × time	[−ΔΔCT] × *t*		n.s.		<1%	n.s.
	macrophages × time	[$CD169^+$, $iron^+/mm^3$] × *t*	−0.000	$p = 0.000003$		<1%	n.s.
Erythropoiesis	$CD34^+CD71^+$ precursors	[log(number/mm^3)][a]		n.s.		<1%	n.s.
	HbA^- NEC (pro-erythrobl.)	[log(number/mm^3)][a]	+0.453	$p < 0.000001$		<1%	n.s.
	HbA^+ NEC (normoblasts)	[log(number/mm^3)][a]	+0.657	$p < 0.000001$		<1%	n.s.
	$p53^+$ NEC	[ratio]		n.s.		<1%	n.s.
	Ery-Is	[log(number/mm^3)]		n.s.		<1%	n.s.
Dependence on time *t*	$CD34^+CD71^+$ cells × time	[log(number/mm^3)] × *t*		n.s.		<1%	n.s.
	HbA^- NEC × time	[log(number/mm^3)] × *t*	−0.257	$p < 0.000001$		<1%	n.s.
	HbA^+ NEC × time	[log(number/mm^3)] × *t*		n.s.		<1%	n.s.
	$p53^+$ NEC × time	[ratio] × *t*		n.s.		<1%	n.s.
	Ery-Is × time	[log(number/mm^3)] × *t*	−0.006	$p < 0.000001$	−0.007	92.69%	$p < 0.000001$
Interaction	Ery-Is × $CD34^+CD71^+$			n.s.		<1%	n.s.
	Ery-Is × HbA^- NEC			n.s.		<1%	n.s.
	Ery-Is × HbA^+ NEC			n.s.		<1%	n.s.
	Ery-Is × $p53^+$ NEC			n.s.	+0.029	4.75%	$p = 0.006$

Note: The effect of Ery-is loss dependent on time. In multivariate analysis, more than 90% of the decline in the Hb value of peripheral blood with time was attributable to a progressive loss of Ery-is. All the other variables (including the total quantity of NEC and the interaction between nuclear p53 protein overexpression of NEC and Ery-is counts) contributed to <10% of progression of anaemia. After start of RBC transfusion(s), the last Hb value prior to transfusion(s) was taken into consideration as long as the Hb value of peripheral blood exceeded that observed prior to transfusion(s). When a Hb value fell below the last Hb value prior to transfusion(s), this lower value was considered. Δ_i is the regression coefficient, LLR the percentage of the change in Hb value determined by the log-likelihood ratio attributable to a variable. $P53^+$ NEC is the proportion of NEC with abnormal nuclear p53 protein overexpression. Further variables, significant neither in uni- nor in multivariate analysis, such as age and sex of patients, transient therapy with ATRA + tocopherol-α, erythropoietin, additional chromosome aberrations, classification, IPSS(-R), are not presented.

Abbreviations: BM, bone marrow; BSC, best supportive care; Ery-is, erythroblastic islands; IPSS-R, Revised International Prognostic Scoring System; LLR, log-likelihood ratio; MDS, myelodysplastic syndrome; MERA, mixed-effects (Poisson) regression analysis; NEC, nucleated erythroid cell; n.s., not significant (i.e. $p > 0.05$); RBC, erythrocyte; *RPS14*, ribosomal protein subunit S14; TD, transfusion dependence; *TNF*-α, tumour necrosis factor α.

[a]Total number of NEC per marrow volume, i.e. total quantity of erythropoiesis within BM.

by immunofluorescence, immunohistochemistry and flow cytometry. Macrophages were marked with F4/80 antibody. NEC were marked by surface markers Ter119 (TER119) and CD71 (C2): RI: $CD71^{high}Ter119^-$; RII: $CD71^{high}Ter119^+$; RIII: $CD71^{intermediate}Ter119^+$; RIV: $CD71^-Ter119^+$. Ery-Is were determined by analogy with human samples. The mouse experiments were performed according to an IACUC-approved protocol at Boston Children's Hospital.

Statistical analyses

The Mann–Whitney *U* test, variance component models (VCM), mixed-effects linear, logarithmic and Poisson regression analyses (MERA),[31–33] and competing-risks regression models were used to test for differences and changes in time relating to the day ($t = 0$) when a patient entered the MDS-003 study. The contribution of a variable to the degree

TABLE 4 Influences on immature erythroid precursors and on Ery-is during BSC of MDS.del(5q) patients with <10% blasts in BM who became RBC-TD ($n = 39$): except for age and sex of patients, and a transient therapy (of 19 patients) with therapy ATRA + tocopherol-α and/or erythropoietin, almost all variables were significant to the numerical densities of $CD34^+CD71^+$ precursor cells and of Ery-is in univariate analyses, but in multivariate analyses, only 1/3 of them remained significant

			$CD34^+CD71^+$ precursor cells/mm^3				Ery-is/mm^3			
			Univariate MERA		Multivariate MERA		Univariate MERA		Multivariate MERA	
Predicted change β_i in the counts (numerical densities) of			β_i	***p* value**	β_i	***p* value**	β_i	***p* value**	β_i	***p* value**
General	Age	On the day of diagnosis (years)		n.s.		n.s.		n.s.		n.s.
	Sex	(male)		n.s.		n.s.		n.s.		n.s.
	Risk score	IPSS-R: (very) low risk (versus other)		n.s.		n.s.	+0.670	$p = 0.016$		n.s.
Genetics	Cytogenetics	% metaphases with del(5q)[a]	+0.014	$p < 0.0005$		n.s.	−0.012	$p < 0.00001$		n.s.
		% interphases with del(5q) (FISH)[a]		n.s.		n.s.	−0.017	$p < 0.00001$		n.s.
		Karyotype: additional aberration(s)[a]		n.s.		n.s.		n.s.		n.s.
	TP53 mutation expression	≥1% marrow cells strongly $p53^+$	−0.025	$p = 0.007$		n.s.	−0.017	$p = 0.00004$		n.s.
		RPS14 mRNA [−ΔΔCT][a]	+0.205	$p = 0.005$		n.s.	+0.149	$p < 0.00001$		n.s.
		TNF-α mRNA [−ΔΔCT][a]	−0.645	$p = 0.05$	−0.201	$p = 0.00016$	−0.035	$p < 0.00001$	−0.088	$p = 0.00008$
		glycophorin A mRNA [−ΔΔCT][a]	+0.198	$p < 0.00001$		n.s.	+0.179	$p < 0.00001$	+0.136	$p < 0.00001$
Haematopoiesis	Cellularity blast excess erythropoiesis	% within bone marrow[a]	+4.596	$p < 0.00001$	+6.748	$p < 0.00001$		n.s.		n.s.
		≥5% (MDS-EB-1)[a]	−1.272	$p = 0.05$		n.s.	−0.917	$p = 0.005$		n.s.
		$CD34^+CD71^+$ precursor cells [/mm^3][a]					+0.003	$p < 0.00001$	+0.008	$p < 0.00001$
		HbA^- NEC [per Ery-I][a]		n.s.		n.s.				
		$p53^+$ NEC [ratio][a]	+0.717	$p < 0.00001$		n.s.	+0.002	$p = 0.004$	+0.003	$p = 0.0006$
		atypias >10% of NEC[a]		n.s.		n.s.	−0.973	$p < 0.00001$		n.s.
		ring sideroblasts >15%		n.s.		n.s.		n.s.		n.s.
		Hb value of blood/HbA^+ NEC[a,b]	−163.0	$p = 0.005$	−383.8	$p < 0.00001$	+3.36	$p < 0.00001$		n.s.
	Macrophages	($CD169^+$) iron-positive [/mm^3][a]	−0.001	$p = 0.013$		n.s.		n.s.		n.s.

TABLE 4 (Continued)

Predicted change β_i in the counts (numerical densities) of			$CD34^+CD71^+$ precursor cells/mm^3				Ery-is/mm^3			
			Univariate MERA		Multivariate MERA		Univariate MERA		Multivariate MERA	
			β_i	*p* value	β_i	*p* value	β_i	*p* value	β_i	*p* value
Other	Length *t* of time	Since diagnosis of disease (years)[a]	−0.193	$p = 0.0008$		n.s.	−0.280	$p < 0.00001$	−0.140	$p = 0.0002$
	therapy	ATRA + tocopherol-α, erythropoietin		n.s.		n.s.		n.s.		n.s.
		Duration of therapy [years][a]		n.s.		n.s.	+0.156	$p = 0.02$		n.s.
		RBC-TD		n.s.		n.s.	−0.684	$p < 0.00001$		n.s.

Note: During the early phase of MDS.del(5q), the numbers of $CD34^+CD71^+$ precursors, but not those of Ery-is were increased inversely proportional to the (RPS14-dependent) efficacy of normoblasts in differentiating to erythrocytes (determined by the ratio of Hb value of blood to the normoblast number in BM). Ery-is loss cumulated (Ery-is/mm^3 declined exponentially with time). The numerical density of $CD169^+$ or iron-positive macrophages (iron accumulation) did not provide predictive significance independently of TNF-α gene expression. β_i = changes in the numerical densities dependent on alterations of the "independent" variables. MF or mutations of genes other than *TP53* were not considered because of too small numbers of patients. A transient therapy with ATRA + tocopherol-α and/or erythropoietin did not influence any of the erythropoietic components.

Abbreviations: ATRA, all-trans retinoic acid; BM, bone marrow; BSC, best supportive care; Ery-is, erythroblastic islands; FISH, fluorescence *in situ* hybridization; IPSS-R, Revised International Prognostic Scoring System; MDS, myelodysplastic syndrome; MERA, mixed-effects (Poisson) regression analysis; NEC, nucleated erythroid cell; n.s., not significant (i.e. $p > 0.05$); RBC, erythrocyte; *RPS14*, ribosomal protein subunit S14; TD, transfusion dependence; *TNF-α*, tumour necrosis factor α.

[a]Included as time-dependent variables (i.e. variables changing with time).

[b]Hb value/l blood relative to the number of HbA^+ NEC/mm^3 BM (i.e. production of erythrocytes by normoblasts).

or progression of anaemia was checked by the method of adequacy determining the proportion of the log-likelihood ratio (LLR) attributable to a variable. A result was considered 'significant' if the (two-sided) probability *p* of the null hypothesis was less than or equal to 0.05.

Further details on methods are presented in the Supporting information.

RESULTS

Anaemia and its progression in MDS.del(5q)

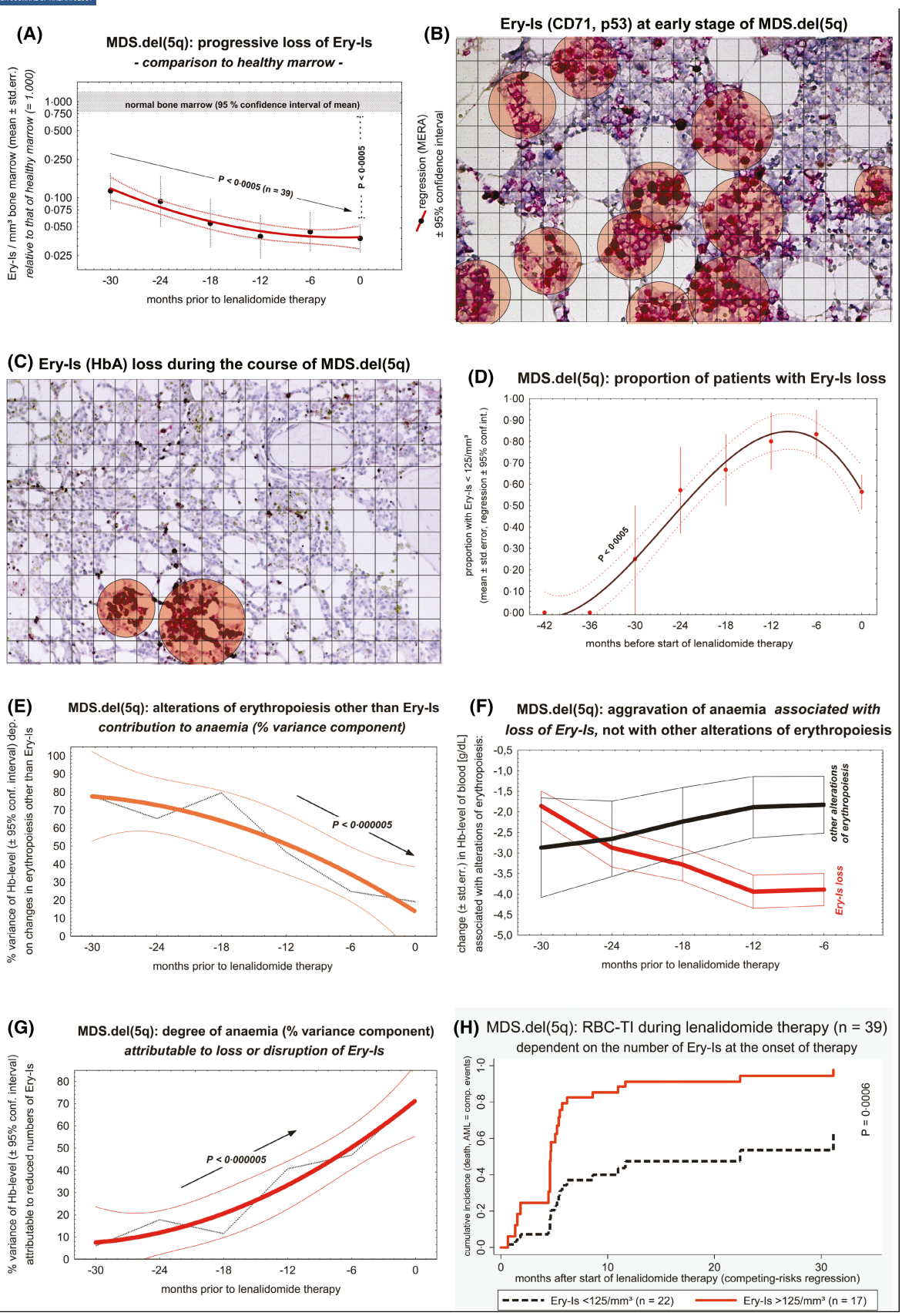
At the early phase of MDS.del(5q), 77.8% of the degree of anaemia was attributable to increased apoptosis and/or nuclear p53 protein overexpression of normoblasts and pro-erythroblasts (Figure 1A, Figure S1A–C), associated with reduced RPS14 mRNA expression of BM cells (Table 2), findings resembling those in the mouse model of *RPS14* haploinsufficiency (Figure S1D).

Anaemia significantly progressed (Figure 1B). This could neither be attributed to the alterations at the level of pro-erythroblasts and normoblasts which did not significantly change during the course of disease (Figure 1C), nor to an increase in the percentages of metaphases or interphases with del(5q) (Figure 1D), nor to a transient therapy with ATRA, tocopherol-α or erythropoietin, nor to any of the other indicators of progressive disease taken into consideration (Figure 1E–G; Table 3; Figure S2). In *TP53*-mutated patients, nuclear p53 protein overexpression of NEC was less obvious (Figure 1H). Blast excess of less than 10%, MF, erythroid/multilineage dysplasia, *TP53* mutation and karyotype aberrations other than del(5q) contributed to less than 20% of the degree of anaemia (Figure 1I; Table 3).

Erythroblastic island loss in MDS.del(5q)

The numerical density of Ery-Is/mm^3 BM fell below the 50% percentile of the controls in all MDS.del(5q) patients. The main alteration associated with aggravation of anaemia was (marked) loss of Ery-Is below the 5% percentile ($p < 0.000005$; Figure 2A–C, Table 3) affecting 72% of patients within three years (Figure 2C,D) independently of all other variables of progression taken into consideration (Table 4; Figure 1C–F; Figure S2). An average decline in blood Hb value by 15–30 g/l was attributable to alterations of erythropoiesis other than Ery-Is loss, 35–45 g/l, i.e. approximately 70% of the degree of anaemia and 93% of anaemia progression, to disappearance of Ery-Is (Figure 2E–G, Table 3; $p < 0.000005$, multivariate analysis). Alterations of erythropoiesis other than Ery-Is contributed to <5% of exacerbation of anaemia (Table 3).

In MDS.del(5q) with marked Ery-Is loss, the probability of RBC transfusion independence (RBC-TI) during lenalidomide therapy was reduced by 50% (Figure 2H) independently of all the other variables determined at MDS-003 study entry (Table 5).


(A)
MDS.del(5q): progressive loss of Ery-Is
- comparison to healthy marrow -
Ery-Is / mm³ bone marrow (mean ± std.err.)
relative to that of healthy marrow (= 1.000)
normal bone marrow (95 % confidence interval of mean)
P < 0·0005 (n = 39)
P < 0·0005
1·000
0·750
0·500
0·250
0·100
0·075
0·050
0·025
-30 -24 -18 -12 -6 0
months prior to lenalidomide therapy
(B)
Ery-Is (CD71, p53) at early stage of MDS.del(5q)
regression (MERA)
± 95% confidence interval
(C) Ery-Is (HbA) loss during the course of MDS.del(5q)
(D)
MDS.del(5q): proportion of patients with Ery-Is loss
proportion with Ery-Is < 125/mm³
(mean ± std error, regression ± 95% conf.int.)
P < 0.0005
1·00
0·90
0·80
0·70
0·60
0·50
0·40
0·30
0·20
0·10
0·00
-42 -36 -30 -24 -18 -12 -6 0
months before start of lenalidomide therapy
(E)
MDS.del(5q): alterations of erythropoiesis other than Ery-Is
contribution to anaemia (% variance component)
% variance of Hb-level (± 95% conf. interval) dep.
on changes in erythropoiesis other than Ery-Is
P < 0·000005
100
90
80
70
60
50
40
30
20
10
0
-30 -24 -18 -12 -6 0
months prior to lenalidomide therapy
(F)
MDS.del(5q): aggravation of anaemia associated with
loss of Ery-Is, not with other alterations of erythropoiesis
change (± std.err.) in Hb-level of blood [g/dL]
associated with alterations of erythropoiesis:
other alterations of erythropoiesis
Ery-Is loss
-0,5
-1,0
-1,5
-2,0
-2,5
-3,0
-3,5
-4,0
-4,5
-5,0
-30 -24 -18 -12 -6
months prior to lenalidomide therapy
(G)
MDS.del(5q): degree of anaemia (% variance component)
attributable to loss or disruption of Ery-Is
% variance of Hb-level (± 95% conf. interval)
attributable to reduced numbers of Ery-Is
P < 0·000005
80
70
60
50
40
30
20
10
0
-30 -24 -18 -12 -6 0
months prior to lenalidomide therapy
(H) MDS.del(5q): RBC-TI during lenalidomide therapy (n = 39)
dependent on the number of Ery-Is at the onset of therapy
cumulative incidence (death, AML = comp. events)
P = 0·0006
0·0 0·2 0·4 0·6 0·8 1·0
0 10 20 30
months after start of lenalidomide therapy (competing-risks regression)
Ery-Is <125/mm³ (n = 22)
Ery-Is >125/mm³ (n = 17)

Factors associated with loss of erythroblastic islands

In MDS.del(5q), Ery-Is loss was not associated with p53 protein overexpression of NEC, nor with an increase in the percentages of del(5q) metaphases or interphases, erythroid dysplasia, subtype, ring sideroblasts, blast excess <10%, TP53 mutation, transient previous therapy with ATRA or erythropoietin, or additional chromosome aberrations (Figures 1C,D and 3A; Figure S2, Table 4), but with a decline in the number of $CD34^+CD71^+$ precursors (Table 4), a variable (numerical density of $CD34^+CD71^+$ precursors within BM) that was significantly increased during the early phase of MDS.del(5q), inversely proportional to the efficacy of normoblasts in differentiating to erythrocytes (Figure 3B,C; Table 4).

Independent predictor of a decline in the $CD34^+CD71^+$ precursor number and of Ery-Is loss was increased TNF-α gene expression of BM cells (Table 4), an alteration associated with an exponential increase in the number of macrophages without adherence to NEC (Figure 3D), inversely related to RPS14 gene expression of marrow cells ($p < 0.002$) and evolving in 72% of patients (Figure 3E). TNF-α protein overexpression was not observed in NEC, but in macrophages (including those of Ery-Is), associated with a loss of their adherence to NEC and, to a lower extent, in endothelial and monocytic cells (Figure 3F).

Erythroblastic island loss in $RPS14^{-/+}$ mice

Ery-Is loss also occurred in $RPS14^{-/+}$ mice. The degree of Ery-Is loss did not differ from that observed during 18 months of follow-up in MDS.del(5q) (Figure 4A–C). In $RPS14^{-/+}$ mice, approximately 70% of Ery-Is loss was associated with increase and pathologic S100A8 protein overexpression of macrophages, less than 20% with S100A8 protein overexpression of RI precursors (Figure 4D–F). No $RPS14^{-/+}$ mice developed blast excess.

Erythroblastic island loss and residual haematopoiesis without del(5q)

An important difference to $RPS14^{-/+}$ mice was the fact that the percentages of marrow metaphases and interphases with del(5q) did not reach 100% in the majority of MDS. del(5q) patients (Figure 1D). Therefore, we tested the hypothesis using a statistical model whether residual haematopoiesis without del(5q) may contribute to the production of erythropoiesis. Assuming a production of erythropoietic components by residual haematopoiesis without del(5q) as observed in the (age-related) control group with healthy marrow, this hypothesis could not be rejected with respect to the production of $CD34^+CD71^+$ precursors, $CD71^+HbA^-$ and HbA^+ NEC (Figure S3A,B; $p > 0.1$). Considering Ery-Is, however, this hypothesis could not be supported. Even if assuming that all Ery-Is are produced by haematopoiesis without del(5q), the numerical densities of Ery-Is were significantly lower than expected from the percentages of metaphases and interphases without del(5q) (Figure S3C; $p < 0.000005$).

DISCUSSION

Current concepts explain evolution and exacerbation of anaemia in MDS.del(5q) by an exhaustion of normoblasts as a result of cell cycle arrest and apoptosis due to intrinsic stabilization and activation of p53 following MDM2 inhibition by free ribosomal proteins.[7,8,12] Exhaustion of pro-erythroblasts correponding to reports on reduced colony-forming unit-erythroid (CFU-e) in MDS.del(5q)[34] seems to be another characteristic alteration which may be p53 protein expression-independent and may (besides an overexpression of S100A8/A9 proteins[12]) result from ribosomal dysfunction impairing protein synthesis and imbalancing (cytotoxic) haeme and globin syntheses, since this defect can be reversed by inhibiting haeme synthesis[35] or applying the translation enhancer L-leucine.[36,37]

The alterations at the level of normoblasts and pro-erythroblasts, however, remained rather constant during the natural course of (lower-risk) MDS.del(5q) accounting for a moderate decline in the Hb value of peripheral blood by about 20–30 g/l in mean, insufficiently explaining the progress of anaemia as a leading symptom of MDS.del(5q).[14]

The main factor associated with evolution of severe (RBC-TD) anaemia was a third characteristic feature of MDS.del(5q) not reported until now, a marked loss of Ery-Is. It occurred prior to or independently of blast excess

FIGURE 2 Loss of erythroblastic islands (Ery-is), influences on the degree of anaemia and the probability of erythrocyte transfusion independence (RBC-TI) during lenalidomide therapy: During the course of MDS.del(5q), >90% of Ery-Is disappeared in >70% of patients (A–D; B: anti-CD71/anti-p53 double staining, magnification 250×; C: Anti-HbA, magnification 125×). At the early phase of MDS.del(5q), a moderate degree of anaemia was associated primarily with ribosomal protein subunit S14 (RPS14)-dependent alterations of normoblasts and pro-erythroblasts (E) which did not significantly change during the further course of MDS.del(5q) (F). Aggravation of anaemia was related to a progressive loss of Ery-Is (F) which became the leading alteration in the *evolution of severe* anaemia (F, G). The probability of RBC-TI during lenalidomide therapy was reduced by 50% in MDS.del(5q) patients with marked Ery-Is loss (at MDS-003 study entry; H) [E–G: multivariate mixed-effects (Poisson) regression analysis (MERA); H: competing-risks regression analysis]

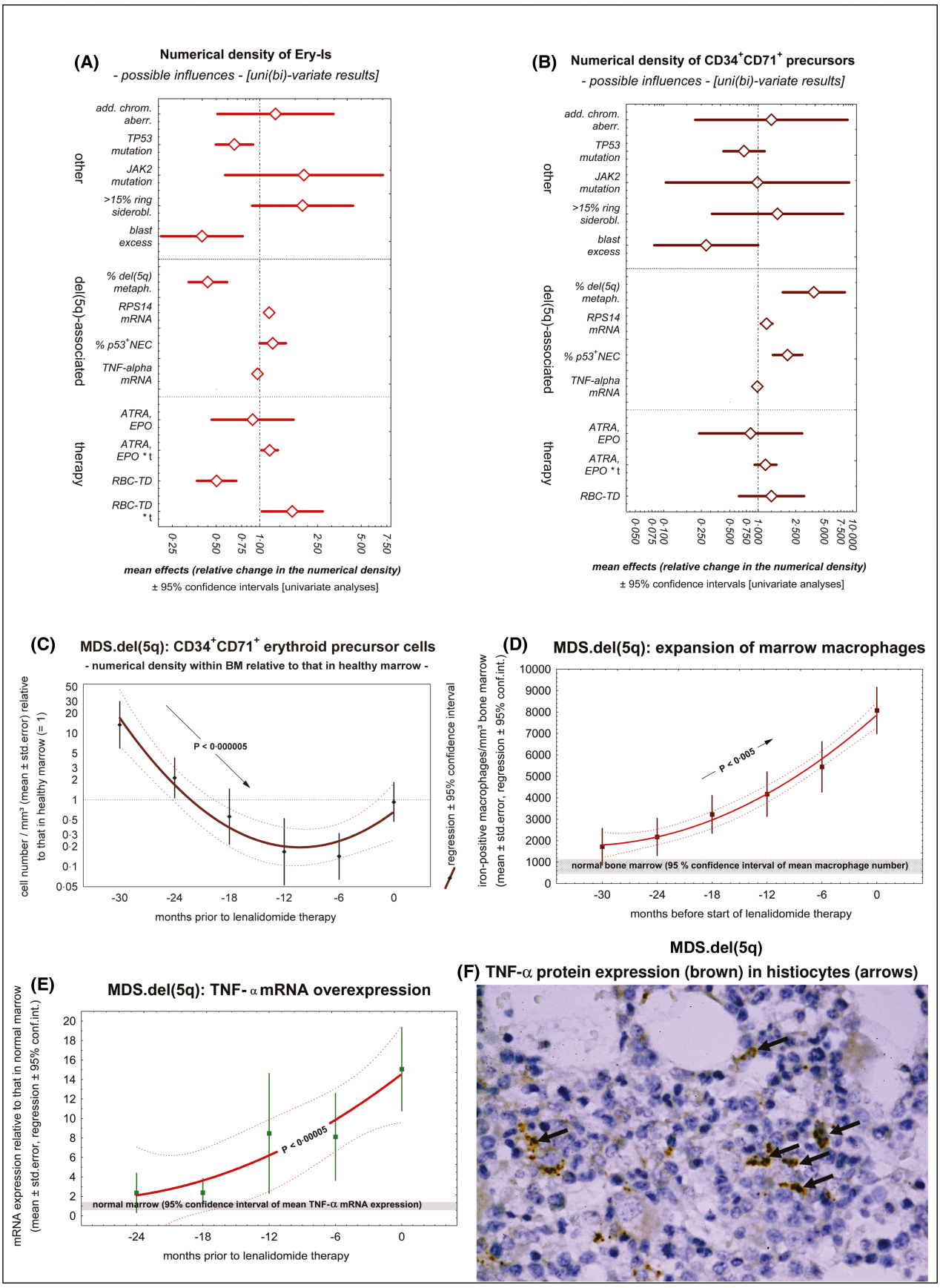
TABLE 5 Prediction of RBC-TI during during lenalidomide therapy calculated from the day of inclusion in the MDS-003 study (RBC-TD IPSS-low- or -intermediate-1-risk MDS.del(5q), n = 39): disruption or loss of Ery-is prior to lenalidomide treatment independently predicted a reduced probability of RBC-TI during lenalidomide therapy

Probability of RBC-TI during lenalidomide therapy in dependence on alterations at MDS-003 study inclusion (competing-risks regression; death and AML = competing events)			Univariate analyses		Multivariate analysis	
			SHR	*p*-value	SHR	*p*-value
General	Age	On the day of study inclusion [years]		n.s.		
	Sex	[male]		n.s.		
	Risk score	IPSS-R: (very) low risk versus other		n.s.		
		WPSS		n.s.		
Genetics	Cytogenetics	% metaphases with del(5q)		n.s.		
		% interphases with del(5q) (FISH)		n.s.		
		Karyotype: additional aberration(s)		n.s.		
	TP53 mutation expression	≥1% marrow cells strongly $p53^+$		n.s.		
		RPS14 mRNA [−ΔΔCT]	1.131	$p = 0.01$		n.s.
		TNF-α mRNA [−ΔΔCT]		n.s.		
		Glycophorin A mRNA [−ΔΔCT]	1.153	$p = 0.002$		n.s.
Peripheral blood	Hb value	Minimum value prior to transfusions [g/l]		n.s.		
	Neutrophils	[10^9/l]		n.s.		
	Platelets	[10^9/l]	1.004	$p = 0.0007$	1.003	$p = 0.007$
	Blasts	[%]		n.s.		
	LDH	[g/dl]		n.s.		
Haematopoiesis	Cellularity blast excess erythropoiesis	% within bone marrow	0.945	$p = 0.02$		n.s.
		≥5% (MDS-EB-1)	0.327	$p = 0.03$		n.s.
		% NEC among cells within BM	1.070	$p = 0.0007$		n.s.
		$CD34^+CD71^+$ precursors [log(counts/mm^3)]		n.s.		
		Ery-Is [log(number/mm^3)]	1.014	$p = 0.0006$	1.011	$p = 0.03$
		HbA^- NEC [log(counts per Ery-I)]		n.s.		
		HbA+ NEC [log(counts per Ery-I)]	1.027	$p = 0.04$		n.s.
		$p53^+$ NEC [ratio]		n.s.		
		atypias >10% of NEC		n.s.		
		ring sideroblasts >15%		n.s.		
	Macrophages	($CD169^+$) iron-positive [/mm^3]		n.s.		
Other	Length *t* of time therapy	Since diagnosis of disease [years]		n.s.		
		ATRA + tocopherol-α, erythropoietin		n.s.		
		duration of therapy [years]		n.s.		

Note: Except for the thrombocyte count of peripheral blood, all the other variables taken into consideration did not provide additional significant information.

Abbreviations: AML, acute myeloid leukaemia; ATRA, all-trans retinoic acid; BM, bone marrow; Ery-is, erythroblastic islands; FISH, fluorescence *in situ* hybridization; IPSS-R, Revised International Prognostic Scoring System; LDH, lactate dehydrogenase; MDS, myelodysplastic syndrome; NEC, nucleated erythroid cell; n.s., not significant (i.e. $p > 0.05$); RBC, erythrocyte; *RPS14*, ribosomal protein subunit S14; SHR, subdistribution hazard ratio; TD, transfusion dependence; TI, transfusion independence; *TNF*-α, tumour necrosis factor α; WPSS, WHO classification-based Prognostic Scoring System.

FIGURE 3 Alterations associated with erythroblastic island (Ery-is) loss: Alterations of the numerical density of Ery-is were associated mainly with alterations associated with del(5q) (A). The narrowness of the confidence intervals of the effects of with ribosomal protein subunit S14 (RPS14) and, tumour necrosis factor α (TNF-α) gene expression indicates their significance for the numerical densities of Ery-is and $CD34^+CD71^+$ precursors (A, B). Additional chromosome aberrations, *JAK2* mutation, ring sideroblasts, blast excess, *TP53* mutation or a transient therapy with all-trans retinoic acid (ATRA) + tocopherol-α or erythropoietin (**t* = adjusted to the period of time since diagnosis of MDS) did not significantly influence the numerical density of Ery-is, nor of $CD34^+CD71^+$ precursors (A, B). Prior to erythrocyte transfusion dependence (RBC-TD), the numerical density of Ery-is was significantly higher. However, when adjusting for the period of time *t* since diagnosis of MDS (RBC-TD**t*), RBC-TD was not an independent predictor of a loss of Ery-Is (A). In contrast to Ery-is, the numerical density of $CD34^+CD71^+$ immature erythroid precursors was increased inversely to the percentage of cells with del(5q), the degree of *RPS14* haploinsufficency and the percentage of p53 overexpressing nucleated erythroid cells (NEC, B). In multivariate analysis, however, impaired formation or loss of Ery-is were associated with a reversal of an initial increase in the number of $CD34^+CD71^+$ erythroid precursor cells (C) as well as an increase in the number of marrow macrophages not showing adhesion to erythroid precursors (D), a finding associated with abnormal TNF-α mRNA and protein overexpression of marrow macrophages (E, F)


(A)
Numerical density of Ery-Is
- possible influences - [uni(bi)-variate results]
add. chrom. aberr.
TP53 mutation
JAK2 mutation
>15% ring siderobl.
blast excess
% del(5q) metaph.
RPS14 mRNA
% p53+NEC
TNF-alpha mRNA
ATRA, EPO
ATRA, EPO * t
RBC-TD
RBC-TD * t
other
del(5q)-associated
therapy
0·25
0·50
0·75
1·00
2·50
5·00
7·50
mean effects (relative change in the numerical density)
± 95% confidence intervals [univariate analyses]
(B)
Numerical density of CD34+CD71+ precursors
- possible influences - [uni(bi)-variate results]
add. chrom. aberr.
TP53 mutation
JAK2 mutation
>15% ring siderobl.
blast excess
% del(5q) metaph.
RPS14 mRNA
% p53+NEC
TNF-alpha mRNA
ATRA, EPO
ATRA, EPO * t
RBC-TD
other
del(5q)-associated
therapy
0·050
0·075
0·100
0·250
0·500
0·750
1·000
2·500
5·000
7·500
10·000
mean effects (relative change in the numerical density)
± 95% confidence intervals [univariate analyses]
(C)
MDS.del(5q): CD34+CD71+ erythroid precursor cells
- numerical density within BM relative to that in healthy marrow -
cell number / mm³ (mean ± std.error) relative to that in healthy marrow (= 1)
regression ± 95% confidence interval
P < 0·000005
50
30
20
10
5
3
2
1
0·5
0·3
0·2
0·1
0·05
-30
-24
-18
-12
-6
0
months prior to lenalidomide therapy
(D)
MDS.del(5q): expansion of marrow macrophages
iron-positive macrophages/mm³ bone marrow (mean ± std.error, regression ± 95% conf.int.)
P < 0·005
normal bone marrow (95 % confidence interval of mean macrophage number)
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-30
-24
-18
-12
-6
0
months before start of lenalidomide therapy
MDS.del(5q)
(E)
MDS.del(5q): TNF-α mRNA overexpression
mRNA expression relative to that in normal marrow (mean ± std.error, regression ± 95% conf.int.)
P < 0·00005
normal marrow (95% confidence interval of mean TNF-α mRNA expression)
20
18
16
14
12
10
8
6
4
2
0
-24
-18
-12
-6
0
months prior to lenalidomide therapy
(F) TNF-α protein expression (brown) in histiocytes (arrows)

less than 10%, *TP53* mutation, MF, risk score, or additional chromosomal aberrations and seems to be a delayed consequence of reduced mRNA expression of the *RPS14* gene since the decline in the number of Ery-Is observed in 18-month-old RPS14$^{-/+}$ mice was indistinguishable from the decline occurring within 18 months of follow-up in MDS.del(5q) patients.

Ery-Is loss could not be attributed to RPS14-, p53- and/or S100A8-dependent alterations of normoblasts or pro-erythroblasts. The factor promoting Ery-Is loss was an expansion of marrow macrophages not showing the adhesion to erythroid cells detectable in healthy marrow, an alteration associated with the *TNF-α* gene and protein overexpression of marrow cells, especially of (CD169^{+}) macrophages, inversely related to *RPS14* mRNA expression, associated with a reversal of an initial increase of CD34^{+}CD71^{+} precursors and resembling findings on an age-related inflammatory BM microenvironment in mice with dual deficiency of *mDia1* and *miR-146a*, genes encoded on the CDR of del(5q).[38] TNF-α is a powerful repressor of erythropoiesis,[39–41] linking MDS.del(5q) to other (congenital) ribosomopathies which are also characterized by TNF-α overproduction of non-erythroid marrow cells.[42]

Our mouse model of *RPS14* gene haploinsufficiency confirmed this relation: about 70% of Ery-Is loss could be attributed to an expansion of S100A8-overexpressing marrow macrophages, about 20% to exhaustion of RI precursors, less than 5% to S100A8 overexpression of NEC. Since S100A8 induces elevated expression of TNF-α by activation of nuclear factor-kappa-B,[43] the relevance of intrinsic (S100A8) and extrinsic (TNF-α) alterations of marrow macrophages to Ery-Is loss in MDS.del(5q) is supported by this animal model.

Considering the relevance of marrow macrophages[44–48] and their adhesion to NEC[49,50] to an adequate function of erythropoiesis,[51–54] a macrophage-associated alteration of the erythropoietic niche, hindering its compensatory increase as well as markedly reducing the overall productive capacity of erythropoiesis, appears to be the leading factor of aggravation of anaemia in MDS.del(5q). The macrophage-associated loss of Ery-Is may furthermore predict reduced efficacy of lenalidomide, the standard therapy of MDS.del(5q)[55] which promotes erythroid differentiation by nuclear translocation of PKC-α.[56] Whether lenalidomide is less effective at the level of the erythropoietic niche should be investigated in further studies evaluating BMBs taken prospectively during this type of therapy.

Although the mouse model of *RPS14* haploinsufficiency indicates the significance of reduced *RPS14* gene expression, there are some differences to human disease:

1. The period of observation is rather short compared to the survival times of patients with MDS.del(5q), indicating that it is a model of early rather than of advanced stage MDS.del(5q).
2. Evolution of iron overload associated with phospholipase-C-beta1-signalling[57] is a characteristic complication of long-standing RBC-TD MDS additionally impairing erythropoiesis. We did not detect an independent (additional) effect of iron accumulation in BM (macrophages) on erythroid failure, but cannot exclude that the periods of follow-up of our patients were too short to uncover this (additional) effect.
3. A dozen genes are known to be mutated in less than 50% of patients with lower-risk MDS.del(5q).[17,19] Since (a) varying degree of Ery-Is loss occurred in all patients and (b) the degree of Ery-Is loss matched to that observed in *RPS14*-haploinsufficient mice by more than 90%, Ery-Is loss in lower-risk MDS.del(5q) may largely be attributable to this haploinsufficiency. If influences other than *RPS14* haploinsufficiency exist, their impact on Ery-Is loss should be significantly lower. Our study was not designed to uncover influences affecting higher-risk or a minority of lower-risk MDS.del(5q) patients, it focussed on the genetic alteration affecting *all* patients with MDS.del(5q), a *RPS14* haploinsufficiency. Nevertheless, *TP53* mutation showed some negative effects on erythropoiesis in univariate (not in multivariate) analyses. From the statistical point of view, the question on possible influences of mutations other than del(5) should be answered by evaluating larger numbers of patients and considering higher-risk MDS.del(5q) (with higher risk of unfavourable gene mutations).
4. The majority of patients with MDS.del(5q) show a relevant proportion of marrow metaphases or interphases without del(5q), indicating that residual haematopoiesis without del(5q) may contribute to the production of erythropoietic cells, (incompletely) antagonizing the effect of del(5q). This assumption could indeed not be disproven with respect to the production of normoblasts, pro-erythroblasts, and CD34^{+}CD71^{+} precursors, but could with respect to the production and formation of Ery-Is, indicating that the S100A8- and TNF-α-associated alteration at the level of marrow macrophages seems to cause a global alteration of the erythropoietic niche concerning both neoplastic haematopoiesis with and residual without del(5q). This might explain the significance of this alteration to aggravation of anaemia in MDS.del(5q) and may support theses on a possible major relevance of pathologic alterations of marrow microenvironment to cytopenias in MDS.[58,59]

Factors associated with anaemia in MDS without del(5q), such as functional inactivation of GATA-1 transcription factor or splicing aberrations following SF3B1 mutation,[2] affect a minority of patients with MDS.del(5q), not sufficiently explaining alterations concerning the great majority of patients, such as Ery-Is loss and RBC-TD anaemia. Haploinsufficiencies of other genes of the CDR, such as *Csnk1a1*, *miRNA145*, *miRNA146a* and *mDia1*, however,

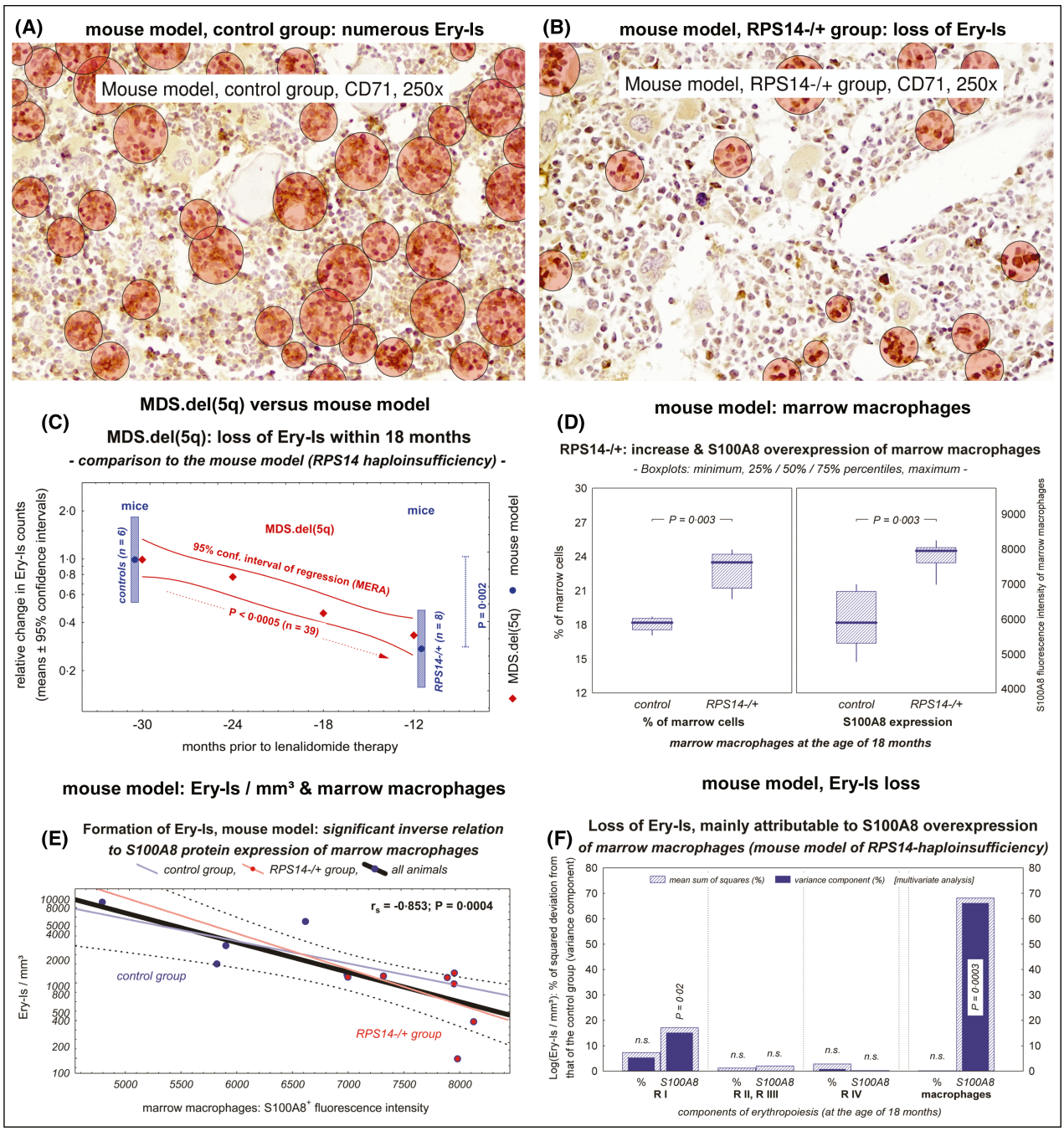


FIGURE 4 Loss of erythroblastic islands (Ery-is) in ribosomal protein subunit S14-negative/positive ($RPS14^{-/+}$) mice: Ery-Is loss also occurred in 18 months old $RPS14^{-/+}$ mice (A, B), and the degree of Ery-is loss resembled the decline detected within 18 months of follow-up in MDS.del(5q)=C: blue = mouse model, red = MDS.del(5q); mixed-effects (Poisson) regression analysis (MERA); r_s = Spearman rank correlation]. In RPS14−/+ mice, Ery-is loss was associated with an abnormal increase in the number of S100A8-overexpressing marrow macrophages (D, E), and about 70% of the degree of Ery-is loss was attributable to this alteration, <20% to S100A8 overexpression of $CD71^{high}Ter119^{-}$ (RI) precursors (F) while S100A8 overexpression of RII, RIII and RIV nucleated erythroid cell (NEC) contributed to <5% of Ery-is loss (F; multivariate MERA)

appear to aggravate anaemia.[38,60] The effects of these haploinsufficiencies on the erythropoietic niche therefore are worth evaluating in further studies.

Thus, in MDS.del(5q), erythroid failure seems to result from a synergy of RPS14-associated deficiencies at the level of normoblasts, pro-erythroblasts, $CD34^{+}CD71^{+}$ precursors, and macrophages with a macrophage-associated failure of the erythropoietic niche as the main alteration in progression of anaemia. To the best of our knowledge, the last finding has not been reported up to now, questioning widespread concepts of anaemia in MDS.del(5q) that do not consider alterations of the erythropoietic niche.[1,2,26]

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CONFLICT OF INTEREST

No competing financial interests in relation to the work described.

AUTHOR CONTRIBUTIONS

Guntram Buesche, the principal investigator, performed histomorphometric and statistical analyses, wrote the manuscript and takes primary responsibility for this paper. Huesniye Teoman performed histomorphometric and immunohistochemical evaluations. Gudrun Göhring and Brigitte Schlegelberger performed cytogenetic analyses. Oliver Bock performed mRNA analyses. Aristoteles Giagounidis, Arnold Ganser, Ulrich Germing, and Carlo Aul recruited and treated patients. Guntram Buesche, Aristoteles Giagounidis, Arnold Ganser, Carlo Aul, and Hans Kreipe classified disease. Rebekka K. Schneider, Flavia Ribezzo, and Benjamin L. Ebert performed the animal model of *RPS14* haploinsufficiency.

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