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Expression of and immune responses to leukemia antigens in patients with hematological malignancies

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Rostock, November 2011

To my family, no words can express my love and gratitude

To Alf, with all my love

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
BAGE	B melanoma antigen
BARD1	BRCA1–associated ring domain 1
BCL-2	B-cell chronic lymphocytic leukemia/lymphoma 2
bcr-abl	Breakpoint cluster region-abelson
BM	Bone marrow
BMMCs	Bone marrow mononuclear cells
BRCA1	Breast cancer 1 early onset
BSA	Bovine serum albumin
CAMs	Cell adhesion molecules
CCL1	CC motif ligand 1
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
CMV	Cytomegalovirus
CR	Complete remission
CSF 1	Colony stimulating factor 1 gene
CTLs	Cytotoxic T lymphocytes
CXCL1	CXC motif ligand 1
DLIs	Donor lymphocyte infusions
DMSO	Dimethyl sulfoxide
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EGR I	Early growth response gene 1
ELISPOT	Enzyme-linked immunospot
ELN	European LeukemiaNet
ERK	Extracellular regulated kinase
FAB	French-American-British
FITC	Fluorescein isothiocyanate

FLT3-ITD	FMS-like tyrosine kinase 3 -internal tandem duplications
FMO	Fluorescence minus one
G250	Carbonic anhydrase 9
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GvHD	Graft versus host disease
GvL	Graft versus Leukemia
HD	Healthy donors
hTERT	Human telomerase reverse transcriptase
IFN-γ	Interferon gamma
IGF I	Insulin-like Growth Factor 1 gene,
IL	Interleukin
IMP	Influenza matrix protein
inv	Inversion
LAAs	Leukemia-associated antigens
LCL	Lymphoblastoid cell line
lGFI-R	Insulin-like growth factor receptor 1 gene
MACS	Magnetic-activated cell sorting
MCP-1	Monocyte chemotactic protein-1
MCs	Mononuclear cells
MDS	Myelodysplastic syndrome
MIF	Macrophage migration inhibitory factor
MIP-1β	Macrophage inflammatory protein
MLPC	Mixed lympocyte peptide culture
MPP11	m-phase phosphoprotein 11
MRD	Minimal residual disease
NPM-1	Nucleophosmin 1
OFA-iLRP	Ovalbumin fetal antigen incomplete laminar receptor protein
OS	Overall survival
PB	Peripheral blood

PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll protein
PR	Partial remission
PR-3	Proteinase-3
PRAME	Preferentially expressed antigen of melanoma
RANTES	Regulated upon activation, normal T-cell expressed, and secreted
RAR-α	Retinoic acid receptor alpha
RD	Refractory disease
RHAMM	Receptor for hyaluronan acid-mediated motility
RQ-PCR	Real-time quantitative reverse-transcriptase polymerase chain reaction
SCT	Stem cell transplantation
SEER	Surveillance Epidemiology and End Results
sICAM-1	Soluble intercellular adhesion molecule-1
sTREM-1	Soluble triggering receptor expressed on myeloid cells
t(;)	Translocation
TAAs	Tumor associated antigens
TAP	Transporter-associated-with-antigen processing
TGF-β	Transforming growth factor
TNF-α	Tumor necrosis factor-alpha
WBC	White blood cells
WHO	World Health Organization
WT1	Wilms' tumor antigen 1

LIST OF FIGURES

LIST OF TABLES

SUMMARY

About 80% of the patients with acute myeloid leukemia (AML) reach complete remission (CR) after chemotherapy. However, more than 50% relapse and only 25% survive longer than five years. Therefore, there is a fervent need for novel therapies to treat leukemia including immunotherapeutic approaches. Leukemic blasts overexpress antigens, so called leukemia-associated antigens (LAAs) like the receptor for hyaluronan acid-mediated motility (RHAMM) and the Wilms' tumor gene 1 product (WT1). These LAAs can be recognized by $CD8^+$ T cells.

In the present study, we analyzed the correlation between the clinical course of 69 patients suffering from leukemia (55 AML/MDS, 6 ALL, 6 CLL and 2 CML) with the expression of RHAMM, WT1 and p53 transcripts before and after treatment, either allogeneic stem cell transplantation (SCT) preceded by chemotherapy or by chemotherapy alone. All gene transcripts were measured by quantitative real time PCR (RQ-PCR) from RNA of peripheral blood mononuclear cell (PBMC) and bone marrow mononuclear cell (BMMC) samples. Furthermore, we determined the presence or absence of spontaneous T cells against RHAMM and WT1 by tetramer staining (flow cytometry) and enzyme-linked immunospot (ELISPOT) assays and correlated them with the outcome of patients. We also wondered whether the cytokine milieu played a role in the favorable outcome of patients (n=8). Therefore we measured diverse cytokines before and after treatment. We hypothesized that RHAMM might serve as a minimal residual disease (MRD) marker, just as reported for WT1 and that the presence of specific T cells may be related to the good outcome of patients, as well as an enriched milieu of cytokines related to T cell proliferation.

After therapy, WT1 transcripts were reduced to normal, as considered as those expressed by healthy donors, and this parameter correlated with a good outcome (*i.e.* CR). The increment of WT1 transcripts eventually resulted in a clinical relapse and subsequent death of the patients. RHAMM did not show a relationship to the clinical status of the patients. Although, we found no significant difference in the presence of cytokines before and after treatment, we observed variations in the cytokine levels in each patient. CD40L and CXCL1 levels increased when the patients remained in CR (50% of the patients), whereas the levels of IL6, IL8, IL17, IL4, IL2, IL1ra, IL1β, IL16, TNF-α, GM-CSF, G-CSF, MIP-1β, CXCL10 and C5a showed a reduction.

Taken together, WT1 is a suitable marker for MRD after allogeneic SCT or chemotherapy. One might speculate that T cells specific for WT1 might contribute to the maintenance of a CR. In contrast, our present study did not support the idea of RHAMM as a MRD marker. Furthermore, specific T cell responses against LAAs, such as RHAMM and WT1 can be raised and these specific CTLs may be raised from cross-reactivity. In addition, an inflammatory and T cell stimulatory cytokine milieu might contribute to the favorable outcome of patients. Relapses predicted by RQ-PCR for WT1 could be prevented by immunotherapy approaches such as antigen-specific donor specific lymphocytes (DLIs) and peptide vaccination.

1 INTRODUCTION

1.1 Overview: Leukemia

Leukemias are clonal disorders of the hematopoiesis starting from leukemia/cancer stem cells. According to their progenitor cell, leukemias are classified as myeloid or lymphoid leukemias. Myeloid leukemias arise from myeloid stem cells, which normally mature into platelets, red or white blood cells. Lymphoblastic leukemias arise from stem cells that will develop into lymphoid progenitors, which normally differentiate into lymphocytes. Acute leukemias (*i.e.* acute myeloid leukemia, AML; acute lymphoblastic leukemia, ALL) progress rapidly and they arise from immature stem cells, while chronic leukemias (*i.e.* chronic myeloid leukemia, CML; chronic lymphocytic leukemia, CLL) progress slowly and arise from more mature stem cells (Horner and Ries 2007).

Leukemias are found among the 10 most common cancers in USA. The median age at diagnosis for leukemia is 66 years of age according to the National Cancer Institute and the incidence (and mortality) varies with ethnicity (Table 1). The Surveillance Epidemiology and End Results (SEER) reported an average incidence of 54.8 per 100,000 people over 65 years of age, whereas in patients younger than 65 years the incidence is 6.0 (Horner and Ries 2007).

Race/Ethnicity	Incidence rates by race		Death rates by race	
	Male	Female	Male	Female
All races	16.1 per 100,000	9.7 per 100,000	9.7 per 100,000	5.4 per 100,000
White	16.8 per 100,000	10.2 per 100,000	10 per 100,000	5.6 per 100,000
Black	12.9 per 100,000	7.8 per 100,000	8.4 per 100,000	5 per 100,000
Asian / Pacific Islander	8.9 per 100,000	6.1 per 100,000	4.9 per 100,000	2.9 per 100,000
American-Indian	9.1 per 100,000	6.5 per 100,000	5.8 per 100,000	3.9 per 100,000
Hispanic	10.8 per 100,000	7.6 per 100,000	6 per 100,000	3.9 per 100,000

Table 1. Incidence and death rates of leukemias considered together. Incidence and death rates of leukemias per ethnicity according to the SEER program of the National Cancer Institute.

1.1.1 Acute myeloid leukemia (AML)

AML is a genetic heterogeneous clonal disorder of the hematopoietic progenitor cells designated as “blasts”. It is the most common myeloid leukemia affecting up to 17.9 per 100,000 adults older than 65 years. The median age at diagnosis is 70 years. Men are affected at a ratio 3:2 when compared to women (Estey and Döhner 2006, Horner and Ries 2007).

AML is characterized by so called class I or class II effect which alters the transcription factor profile (class I) or proliferation (class II) of the hematopoietic stem cells. Either homebox genes, like Cdx2 (Thoene *et al.* 2009), PU.1 and GATA, or proliferation-derived genes, *i.e.* RAS, extracellular regulated kinase (ERK), completely change the character of the stem cells towards malignancy. The genetic damage in AML blasts causes activation of tyrosine kinases, *i.e.* FLT3 and c-KIT,

and the overexpression of HOX genes as a result of the translocation of chromosome 8 and 21, t(8;21), or the inversion of the chromosome 16, inv(16) (Fröhling *et al.* 2005).

AML blasts can be identified for the expression of $CD56^+$, $CD33^+$, $CD13^+$ surface antigens (Estey and Döhner 2006). Organ infiltration by AML blasts is more probable, particularly in brain and lungs, if blasts are $CD54^+$ and the amount of white blood cells (WBC) rises 50,000 (Estey and Döhner 2006, Schmitt *et al.* 2011).

Cytogenetics is an important prognostic factor (Giles *et al.* 2002). Approximately 55% of adults have cytogenetic abnormalities at diagnosis. Based on the cytomorphology and cytochemistry of the AML blasts, the French-American-British (FAB) system has classified AML into eight subtypes (Table 2). This system considers AML when bone marrow has more than 30% blasts. Another system for leukemia classification is provided by the World Health Organization (WHO). The WHO system confirms AML when the bone marrow has 20% blast infiltration. This system is based on cytogenetics and divides AML into four categories. Special cases of AML comprises *i*) acute monocytic leukemia, if more than 80% are monocytes, *ii*) erythroleukemia, if more than 50% of the marrow are normoblasts and if the non-erythroid population has more than 30% myeloblasts, *iii*) megakaryocytic leukemia, if the marrow is unaspirable (Estey and Döhner 2006).

Patients with t(15;17), t(8;21) and inv(16)/ t(16;16) have a favorable prognosis with about 30% risk of relapse. Moreover, trisomy 22 and inv(16) are associated with relapse-free survival. On the other hand, patients with multiple ($\geq$ 3) chromosomal abnormalities, so called complex karyotype, or abnormalities in chromosome 5, 7,

3(q-) or inv(3)/ t(3;3) have a 75% risk of relapse. Furthermore, the deletion of the chromosome Y in men with t(8;21) is associated with shorter survival (Estey and Döhner 2006).

Internal tandem duplications (ITD) in the FMS-like tyrosine kinase 3 (*FLT3*) gene may adversely affect the clinical outcome. Up to 34% of patients with normal karyotype are *FLT3-ITD* positive, and up to 64% have mutations in nucleophosmin 1 (*NPM-1*) gene. Overall survival is better in patients with *NPM1* mutations, but *FLT3-ITD* negative (Estey and Döhner 2006, Giles *et al.* 2002).

AML blasts have different expression of cell adhesion molecules (CAMs) than normal hematopoietic precursors. Such aberrant expression could explain in part the different patterns of trafficking and AML subtypes (Giles *et al.* 2002).

There are diverse factors that may cause AML including ionizing radiation, benzene and chemotherapy, which are related to aberrations in chromosome 5 or 7, or both. However, aberrations in these chromosomes can occur spontaneously and the probability increases with age. Up to 15% of the AML patients developed leukemia after chemotherapy treatment of solid tumors. Chemotherapy-related AML is characterized by either 1) monosomies or deletions of the chromosomes 5q and 7q (-5/-7), normally occurring 5 to 10 years after the exposure to alkylating agents; or 2) cytogenetic abnormalities in the chromosome 11q, t(15;17) and t(8:21). The later emerges in a period from one to five years after chemotherapy (*i.e.* doxorubicin and etoposide, such reagents interact with DNA topoisomerase II). Chemotherapy-related AML, or AML rising after myelodysplastic syndrome (MDS), are usually more resistant than *de novo* AML (Estey and Döhner 2006, Horner and Ries 2007).

Type	Definition	Cytogenetics	Chromosomal imbalances	Genes	Incidence in AML
M0	Minimally differentiated AML	del(5q), del(7q)	+13	EVI1, RPN1	2-3%
M1	AML with few or not mature cells	inv(3)(q21q26) or t(3;3)(q21;q26)	+13, +11	EVI1, RPN1	15-20%
M2	AML with granulocytic maturation	t(8;21)(q22;q22), t(6;9)(p23;q34)	+8, +11	RUNX1, RUNX1T1, ETO, DEK, NUP214	30-40%
M3	Acute promyelocytic leukemia	t(15;17) (q22; q11-21)	Largely absent	PML; RARA	5-10%
M4	Acute myelomonocytic leukemia	inv(16)(p13q22), del(16q) or t(6;11)(q27;q23) or inv(3)(q21q26) or t(3;3)(q21;q26) or t(6;9)(p23;q34)	+8, +22	MLL, AF6, EVI1, RPN1, DEK, NUP214	15-20%
M4Eo	Myelomonocytic together with bone marrow eosinophilia	inv(16)(p13q22) or t(16;16)(p13;q22)	+22	CBFB, MYH11	5%
M5	Acute monoblastic leukemia (M5a) or acute monocytic leukemia (M5b)	del (11q), t(9;11)(p22;q23), t(11;19) or t(6;11)(q27;q23)	+8	MLL, AF9, AF6	10%
M6	Acute erythroid leukemias, including erythroleukemia (M6a) and very rare pure erythroid leukemia (M6b)	inv(3)(q21q26) or t(3;3)(q21;q26)	Not reported	EVI1, RPN1	5%
M7	Acute megakaryoblastic leukemia	t(1;22) or inv(3)(q21q26) or t(3;3)(q21;q26)	+19	EVI1, RPN1	1%

Table 2. AML classification according to FAB. FAB divides AML into 8 subtypes (M0-M7) according to the cytogenetics and maturation of the blast. Subtypes have different responses to therapy. The incidence of each subtype varies being the most common M2 (Alvarez *et al.* 2001, Estey and Döhner 2006, Karnan *et al.* 2006)

AML patients are conventionally treated with chemotherapy. Approximately 70-80% of the patients younger than 60 years reach a complete remission (CR). CR is defined as marrow with less than 5% blast, more than 1,000 neutrophils and 100,000 platelets. However, most of the patients in this stage eventually relapse and die due to the disease or to infections (*e.g.* Candida, Aspergillus) as a consequence of the bone marrow failure and the resulting immune deficiency. Furthermore, only 10% of patients older than 60 years reached CR. Such an event is related to their cytogenetic abnormalities (*i.e.* chromosome 5 and 7) or inability to survive treatment (Estey and Döhner 2006).

Once remission is achieved further treatment is needed to prevent relapse. Patients under 60 years may receive intensive chemotherapy, autologous stem cell transplantation (SCT) or allogeneic SCT (Giles *et al.* 2002, Estey and Döhner 2006). Targets of chemotherapy are FLT3-ITD and RAS signaling pathway (Tallman *et al.* 2005).

Chemotherapy eliminates blasts found in the periphery more effectively than those that reside in the bone marrow (McQueen *et al.* 2005). Probably the hematopoietic microenvironment protects blasts from apoptosis. This interaction may be mediated by CXCR4, expressed on blasts, and its ligand the stromal-cell-derived factor 1 (Lapidot and Kollet 2002).

Allogeneic SCT has become another modality of treatment for patients with high risk leukemia. SCT may improve survival, especially, in patients with adverse prognosis (Weber *et al.* 2009). It may also support long-term remission based on the favorable

graft-versus-leukemia (GvL) effect mediated by T cells. However, it could also induce the noxious graft-versus-host disease (GvHD) in patients after transplantation.

1.1.2 Cytokine milieu

AML blasts produce chemokines that inhibit normal blasts to differentiate physiologically (Estey and Döhner 2006). Furthermore, leukemic blasts secrete IL-1β that stimulates the release of granulocyte macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) from endothelial cells, which generates a positive feedback to the proliferation of leukemic cells. Leukemia cells themselves also produce and secrete GM-CSF and G-CSF (Nara 1993). AML blasts expressing CXCR4 are attracted by the chemokine SDF-1. This mechanism may at least partially explain the infiltration of blast into marrow and tissue in AML M4/M5 subtype (Giles *et al.* 2002). AML blasts also secrete platelet-derived growth factor (PDGF), a mitogen for marrow stromal cells (Giles *et al.* 2002).

1.1.3 Myelodysplastic syndrome (MDS)

MDS is a heterogeneous clonal hematopoietic disorder of the stem cells. The dysplastic hematopoiesis of one or more cell lineages are ineffective in this malignancy. MDS is characterized by cytopenias in the peripheral blood and a high risk of progression into AML. Clinical and biological studies suggested considering MDS and AML as part of the same continuous disease spectrum (Cilloni *et al.* 2003).

MDS classification according to FAB system is summarized in Table 3.

Type	Features
RA	< 5% blast infiltration in BM
RARS	< 5% blast infiltration in BM and ≥ 15% red cell precursors in the marrow (ring sideroblasts)
RAEB-I	> 5-10% blast infiltration in BM
RAEB-II	10-19% blast infiltration in BM
CMML	>20% macrophages and monocytes in BM

Table 3. MDS classification according to FAB. FAB has classified MDS into 5 subtypes according to the percentage of myeloblasts in the bone marrow. **Abbreviations.** BM: bone marrow, RA: refractory anemia; RARS: refractory anemia with ringed sideroblasts, RAEB: refractory anemia with excess blasts, CMML: chronic myelomonocytic leukemia.

1.2 Leukemia-associated antigens (LAAs)

Leukemia blasts overexpress proteins that play an important role in their survival and proliferation, as well as in apoptosis and downregulated-differentiation. Such proteins have been designated leukemia-associated antigens (LAAs).

LAAs comprise a broad group of proteins including Wilms' tumor gene product 1 (WT1), the receptor for hyaluronic acid mediated motility (RHAMM), survivin, ovalbumin fetal antigen incomplete laminar receptor protein (OFA-iLRP), breakpoint cluster region-abelson (bcr-abl), m-phase phosphoprotein 11 (MPP11), proteinase-3 (PR-3), B melanoma antigen (BAGE), carbonic anhydrase 9 (G250), human telomerase reverse transcriptase (hTERT), B-cell chronic lymphocytic

leukemia/lymphoma 2 (BCL-2), FLT3-ITD, preferentially expressed antigen of melanoma (PRAME), among others (Casalegno-Garduño *et al.* 2011).

WT1, RHAMM and PR-3 have been identified to specifically generated T cell responses in AML patients (Oka *et al.* 2004, Rezvani 2008, Schmitt *et al.* 2008). Therefore these antigens are aimed for immunotherapeutic approaches (Casalegno-Garduño *et al.* 2011). These antigens are HLA-A2 restricted, approximately 40% of the general population is HLA-A2 positive (Estey and Döhner 2006).

1.2.1 Wilms' tumor gene product 1 (WT1)

WT1 gene was initially identified as mutated in nephroblastoma, also known as Wilms' tumor, a common pediatric kidney cancer (Call *et al.* 1990). *WT1* is located at the chromosome locus 11p13. It comprises 10 exons and codifies for a 52-55 kDa protein with four zinc fingers that functions as a transcription factor (Bergmann *et al.* 1997a, Casalegno-Garduño *et al.* 2010).

WT1 is required for normal embryonic development as demonstrated by the inability of homozygous $WT1^{-/-}$ mouse embryos to survive. It is involved in the ontogenesis of the urogenital system (kidneys and gonads), as well as in the development of brain, olfactory epithelium, adrenal glands, mesothelial tissue, retina and spleen of mammals (Armstrong *et al.* 1993, Wagner *et al.* 2005). It is expressed transiently in $CD34^{+}$ cells of early haematopoiesis and mesenchymal cells, and at low levels in some tissues such as testis, placenta, ovaries, myometrium, stromal cells of the uterus, brain, heart, lung, intestine, liver, spleen and kidney (Bergmann *et al.* 1997a,

Schmid *et al.* 1997, Cilloni and Saglio 2004, Greiner *et al.* 2004, Wagner *et al.* 2005, Greiner *et al.* 2006, Hosen *et al.* 2007, Cilloni *et al.* 2008, Greiner *et al.* 2008).

1.2.1.1 The function of WT1

WT1 functions as a repressor of genes related to apoptosis, growth and differentiation (Bergmann *et al.* 1997a, Clark *et al.* 2007, Greiner *et al.* 2008). Target genes include *p21*, *E-cadherine*, *CSF-1*, *IGF-II*, *IGF-IR*, *EGR1*, *PDGF*-A, *TGF-β*, *bcl-2*, *c-myc*, *PAX22*, retinoic acid receptor (*RAR*)-*α*, *syndecan-1*, *amphiregulin* and in a reciprocal manner the tumor suppressor *p53* and itself (Maheswaran *et al.* 1993, Bergmann *et al.* 1997a, Englert *et al.* 1997, Cilloni and Saglio 2004, Clark *et al.* 2007). It is also involved in RNA metabolism and in the progression of the cell cycle (Ito *et al.* 2006).

During the differentiation of the cells, WT1 is down-regulated (Bergmann *et al.* 1997a, Wagner *et al.* 2005, Ito *et al.* 2006). Inhibition of WT1 with antisense oligomers leads to differentiation, decreased proliferation, growth arrest and apoptosis (Inoue *et al.* 1998, Algar *et al.* 1996). Therefore the expression of WT1 is indispensable for tumor proliferation.

1.2.1.2 WT1 isoforms

Four different isoforms result from the alternative splicing of the gene at two different sites. The insertion or omission of 17 amino acids (from exon 5) between the transactivating and the zinc finger domain leads to one splice variant. The other alternate splicing event is the result of the presence or absence of the KTS tripeptide, Lysin-Threonin-Serin, located at the end of exon 9. Insertion of KTS separates zinc finger 3 and 4, thus altering subcellular location, backbone flexibility and DNA-binding. The –KTS isoform binds DNA with higher affinity than +KTS isoform. Isoforms are expressed at different ratios, 8.3: 3.8: 2.5:1 for +17AA+KTS, -17AA+KTS, +17AA-KTS and -17AA-KTS; respectively. Developmental abnormalities have been reported in patients with altered ratios. All of the four isoforms are expressed in primary human solid tumors (*i.e*. breast tumor, lung cancer, sarcoma, HNSCC) and primary leukemia (Bergmann *et al.* 1997a, Laity *et al.* 2000, Ito *et al.* 2006, Burwell *et al.* 2007).

Each WT1 isoform has specific paradoxical functions, either as a tumor suppressor as well as an oncogene. Isoforms lacking exon 5 causes slowed proliferation, whereas isoforms containing exon 5 alters cellular morphology (Hewitt and Sanders, 1996). Therefore, it is suggested that the anti-apoptotic role of WT1 relies on the 17AA region and it exerts its function through stabilization of the mitochondria membrane activity. More specifically, the +17AA-KTS isoform is involved in tumorigenesis of lymphoid malignancy (Li *et al.* 2003, Ito *et al.* 2006) and in the increased expression of the anti-apoptotic gene *bcl-2* and in the suppression of the proapoptotic gene *bak* (Ito *et al.* 2006). The +17AA+KTS isoform has characteristics

of an oncogene, whereas the -17AA-KTS isoform has features of a tumor suppressor (Englert *et al.* 1997, Loeb *et al.* 2003, Ito *et al.* 2006, Burwell *et al.* 2007, Tatsumi *et al.* 2008). The function of -17AA+KTS isoform is unknown so far.

Chemotherapeutic reagents (*i.e.* etoposide, doxorubicin) initiate cell death by triggering mitochondrial apoptotic pathways. Therefore, targeting exon 5 of WT1 could serve as an anti-cancer agent (Ito *et al.* 2006, Tatsumi *et al.* 2008)

1.2.1.3 WT1 and leukemia

WT1 does not appear to be expressed by normal cells at the adult stage (Cilloni *et al.* 2008, Cilloni *et al.* 2009). On the other hand, it is overexpressed in a broad variety of solid tumors, *i.e.* Wilms' tumor, melanoma, ovarian cancer, lung cancer, breast cancer, colorectal carcinoma, thyroid, HNSCC, brain tumors, as well as in leukemias (AML, ALL, CML, MDS) (Cilloni *et al.* 2003, Greiner *et al.* 2003, Greiner *et al.* 2006, Ito *et al.* 2006, Clark *et al.* 2007, Greiner *et al.* 2008, Casalegno-Garduño *et al.* 2011). In animal models, bone marrow cells with high expression of WT1 tend to become leukemic (Osaka *et al.* 1997)

The expression of WT1 in leukemias was first described by Miwa *et al.* (1992). *WT1* mRNA is expressed in 80% (ranging from 44% to 100%) of reported cases of both AML and ALL (Miwa *et al.* 1992, Brieger *et al.* 1994, Menssen *et al.* 1995), whereas WT1 protein is expressed in 60% (Menssen *et al.* 1995). Its overexpression correlates with the blast percentage in the bone marrow and peripheral blood (Cilloni *et al.* 2008, Bergmann *et al.* 1997a).

High levels of *WT1* mRNA correlate with worse long-term outcome in AML patients (Bergmann *et al.* 1997b). Less *WT1* transcripts are found in the AML subtype M5 than in other subtypes of AML (Miwa *et al.* 1992, Bergmann *et al.* 1997a, Bergmann *et al.* 1997b). It is expressed in blast crisis CML but not during the chronic phase (Bergmann *et al.* 1997a).

In an extensive cohort, Gaidzik *et al.* (2009) reported 13% (78 of 617) of the AML patients have mutations of WT1. Mutations were detected in exon 7 (54 of 78), exon 9 (13 of 78) and in exons 1, 2, 3 and 8. Such mutations were associated with younger age and the presence of FLT3-ITD. However mutations on WT1 had no effect on the survival of the patients when compared to wild type-WT1 patients. Nevertheless if patients were FLT3-ITD$^+$ and had mutated WT1, then they had lower CR rate and relapse-free and overall survival (Bergmann *et al.* 1997a, Gaidzik *et al.* 2009).

In general terms, AML patients that achieved CR are WT1 negative, whereas persistence of WT1 indicates treatment failure (Bergmann *et al.* 1997a, Cilloni *et al.* 2008). However, most of the patients in remission have residual AML blasts that eventually lead to relapse (Estey and Döhner 2006). Thereafter the prediction of MRD may prevent a relapse by salvage treatment. WT1 was proposed as a MRD marker since early 1990's in AML (Inoue *et al.* 1994, Inoue *et al.* 1996, Bergmann *et al.* 1997a, Bergmann *et al.* 1997b, Sugiyama 1998) and confirmed by diverse groups later on (Cilloni and Saglio 2004, Cilloni *et al.* 2008, Cilloni *et al.* 2009, Nowakowska-Kopera *et al.* 2009). It has also been proposed as a marker of MRD in MDS (Cilloni

et al. 2003). Its expression can be detected from weeks up to three months before the clinical relapse is observed (Bergmann *et al.* 1997a, Cilloni *et al.* 2009).

1.2.1.4 Immune responses against WT1

WT1 induces spontaneous immune responses in AML patients (Greiner *et al.* 2008). Oka *et al.* (2004) found spontaneous HLA-A2-restricted WT1-specific cytotoxic T lymphocytes (CTLs). Epitopes of WT1 (*i.e.* RMF PNA PYL) are recognized by $CD8^+$ T cells and $WT1^+$ AML blasts are lysed in a dose-dependent manner. This response positively correlates with the decreased transcripts of the gene and may contribute to the favorable outcome of patients. Remarkably, WT1-specific T cells are able to lyse $WT1^+$ tumor cells *in vitro,* but not $WT1^+$ normal cells (Oka *et al.* 2000a, Oka *et al.* 2000b). Moreover, spontaneous humoral responses have been found against WT1 product in AML, MDS and CML (Gaiger *et al.* 2001, Nicoli *et al.* 2008). In contrast the levels of anti-WT1 antibodies in healthy donors are very low, implicating that the low amount of antigen present in healthy donors is unable to stimulate a significant humoral response (Nicoli *et al.* 2008).

Immunotherapy using WT1 peptides in clinical vaccination trials has shown positive effects in patients (Mailänder *et al.* 2004, Oka *et al.* 2004, Keilholz *et al.* 2009, Maslak *et al.* 2010). Vaccination with either native or analogue epitopes generated specific CTLs. Analogue peptides differ in one of the amino acids of the native sequence and can generate stronger responses than native peptides (Maslak *et al.* 2010).

The new avenue in clinical trials is the use of WT1 peptides to stimulate both $CD8^+$ and $CD4^+$ T cell responses (Maslak *et al.* 2010). Long peptides are more efficient than class I epitope peptides. This new approach induces synergic effects, supporting prolonged immune responses.

Another immunotherapeutic approach is to isolate WT1-specific T cells from peripheral blood of healthy donors and to expand them through *in vitro* stimulation (Weber *et al.* 2009). These T cells remain functionally active and are able to lyse $WT1^+$ tumor cells lines. Such T cells could be used as adoptive immunotherapy after allogeneic SCT in AML patients or during the CR of patients with adverse prognosis.

Combined therapy is another approach to treat leukemias. CML patients treated by imatinib have been vaccinated with analogue WT1 peptides. WT1-specific T cells were detected in the peripheral blood and those inversely correlated with *bcr-abl*. WT1 specific CTLs can be detected in a long term period even after the cessation of the vaccine (Narita *et al.* 2010, Oji *et al.* 2010).

1.2.2 The receptor for hyaluronan acid-mediated motility (RHAMM)

RHAMM is located on the human chromosome band 5q33.2 (Spicer *et al.* 1995) and contains 18 exons. In humans the resulting product of the full-length mRNA is a protein of 85 kDa. Moreover, RHAMM has multiple alternative spliced variants lacking exon 4 or exon 13, which results in the shorting of 45 bp or 147 bp; respectively. Another variant lacks both exons. The full-length RHAMM has five

functional domains, designated D1-D5. The D2-D5 domains are required for cell motility and passage through the cell cycle, whereas the D1-domain is the down regulator of the protein. Therefore the full-length is an inactivated form of RHAMM. Truncated forms of RHAMM, 60-73 kDa, are expressed after tissue injury, as well as in tumors. These forms are active forms of the protein (Turley and Harrison 1999, Turley *et al.* 2002).

RHAMM, also designated CD168, was first isolated from supernatant of non-confluent hearth-fibroblast of chicken embryos. It was described as a soluble binding protein (Turley 1982).

1.2.2.1 Functional aspects of RHAMM

Hyaluronan is the counter-ligand of RHAMM, therefore making RHAMM part of the heterogeneous group of the hyaladherins. The result of the binding of RHAMM to hyaluronan promotes normal wound healing, motility, adhesion, proliferation, migration and angiogenesis (Till *et al.* 1999, Tolg *et al.* 2006, Slevin *et al.* 2007, Gao *et al.* 2008). It is also involved in transformation, metastasis, invasion, growth, modification of the RAS signaling cascade and progression of inflammatory diseases such as arthritis in animal models (Hall *et al.* 1995, Nagy *et al.* 1995, Hall *et al.* 1996, Naor *et al.* 2007, Buganim and Rotter 2008). Additionally, inhibition of RHAMM leads to mitotic arrest (Mohapatra *et al.* 1996).

One of the best documented functions of RHAMM is its role in wound healing, this occurs in response to hypoxia and TGF-β1 (Samuel *et al.* 1993). Moreover, this mechanism is used by tumor cells (Maxwell *et al.* 2008).

RHAMM is regulated by TGF-β1, FGF, β1 integrins, PKC and H-RAS (Buganim and Rotter 2008). *In vitro* studies showed that the tumor suppressor p53 down-regulates *RHAMM* mRNA in human colorectal adenocarcinoma (Sohr and Engeland 2008). However, p53 is mutated in less than 20% of the leukemia patients. Therefore, it is assumed that there is a defect in the down- or upstream of p53 cascade (Boyapati *et al.* 2004, Prokocimer and Peller 2011).

RHAMM expression varies during the cell cycle; the mRNA maximum expression is observed during mitosis, whereas the protein expression reaches a peak at the S phase (Sohr and Engeland 2008). The expression of RHAMM is not essential neither during the embryonic development nor normal adult homeostasis of mice.

RHAMM is located at different compartments of the cell including cell surface, cytoskeleton, mitochondria and nucleus. Therefore, it has been suggested to be involved in the exchange of information between the cell genome and the extracellular environment, an event denominated dynamic reciprocity (Turley *et al.* 2002). In general terms, proteins that have dual functions, as cell-surface as well as cytoplasmic, are related to the stress-response by cells, and tumor cells use this adaptive mechanism. Both intracellular and extracellular isoforms of RHAMM have been related to cancer (Maxwell *et al.* 2008)

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1.2.2.2 RHAMM isoforms

The intracellular isoform plays a role in the assembly of the cytoskeleton and mitotic spindle, as well as in the maintenance of the centrosome integrity, cell cycle progression from G2 to mitosis, signaling, tumorigenesis and cell proliferation (Maxwell *et al.* 2003, Buganim and Rotter 2008, Sohr and Engeland 2008). To this end it associates with kinases, calmodulin, actin filaments, interphase microtubules, mitotic spindle microtubules, centrosome, podosomes and with genes related to DNA repair (Maxwell *et al.* 2003, Sohr and Engeland 2008). It influences tumor progression through the binding of the mitotic spindle and centrosome (Maxwell *et al.* 2008).

Intracellular RHAMM proteins may act as adapter molecules through their association with kinases, such as ERK1 (Turley and Harrison 1999). RHAMM is regulated by the breast cancer 1 early onset (BRCA1) –associated ring domain 1 (BARD1) (Pujana *et al.* 2007). Mutations on *BARD1* lead to genetic susceptibility to breast, ovarian and prostate cancers (Maxwell et al, 2008).

Some groups suggested that the nuclear-located RHAMM function as a tumor suppressor, since its normal expression is required for mitotic spindle, centrosome integrity and genomic stability, and it is expressed in a cell-cycle dependant manner (Godar and Weinberg 2008, Sohr and Engeland 2008). The aberrant mitotic assembly results if RHAMM is overexpressed or if the RHAMM-spindle interaction is inhibited, particularly in the absence of BARD1 (Maxwell *et al.* 2008). High

expression of RHAMM correlates with genomic instability in multiple myeloma (Maxwell *et al.* 2004).

The extracellular RHAMM isoforms result from a redistribution of the intracellular isoform, though not necessarily associated to an increased synthesis of mRNA or protein (Maxwell *et al.* 2008). Cytoplasmic RHAMM is exported to the cell surface by unconventional mechanisms in response to specific stimuli. In general terms, the non-conventional export of proteins can increase in cellular transformation and in neoplastic progression (Turley *et al.* 2002, Maxwell *et al.* 2008). The export of cytoplasmic RHAMM to the cell surface specifically associates with cell transformation in multiple myeloma (Adamia *et al.* 2005), inflammatory disorders (Nedvetzki *et al.* 2004) and wound healing (Tolg *et al.* 2006).

Once in the extracellular media RHAMM pairs with CD44, since RHAMM lacks both a signal peptide and a transmembrane domain (Turley *et al.* 2002). In *in vitro* studies, the extracellular RHAMM-CD44 complex coordinates invasion and migration in aggressive breast cancer cell lines (Hamilton *et al.* 2007). RHAMM-CD44 complex controls signaling through RAS proteins, which commonly are mutated in human cancers. Interestedly, CD44 itself is a receptor that can promote invasion and metastasis in experimental tumor models. Also, it is present in aggressive tumor progenitor cell subsets in leukemia, breast and prostate cancers (Maxwell *et al.* 2008).

Extracellular RHAMM is required after stretch injury, motility, progression of cell cycle and transformation (Turley *et al.* 2002).

1.2.2.3 Expression of RHAMM in normal tissue and in cancer

The full length RHAMM is constitutively expressed in most normal human cell types including fibroblasts, smooth muscle cells, endothelial cells, macrophages, immature thymocytes, B cells, stromal cells from the bone marrow, keratinocytes, sperm, astrocytes, astrocytomas, nerve cells of the central system and olfactory system, microglia, oligodendrocytes and cells from stomach, testis, endometrium and placenta (Turley and Harrison 1999, Rein *et al.* 2003). Shorter forms of RHAMM transiently appear in normal tissue in response to injury. Moreover, some normal tissue dividing actively had elevated expression of RHAMM (Line *et al.* 2002).

On the other hand, multiple forms of RHAMM are overexpressed in a broad variety of solid tumors such as breast cancer (Assmann *et al.* 1998), pancreatic cancer (Abetamann *et al.* 1996), colon, stomach cancer (Li *et al.* 2000), gastric cancer, endometrial carcinoma (Rein *et al.* 2003), oral squamous cell carcinoma, squamous cell lung carcinoma (Teder *et al.* 1995), and malignant melanoma (Ahrens *et al.* 2000), as well as in leukemia including AML, B-CLL and multiple myeloma (Crainie *et al.* 1999, Greiner *et al.* 2002). Seventy percent of AML patients overexpressed RHAMM at both mRNA and protein level (Greiner *et al.* 2002, Greiner *et al.* 2003). However, there is new evidence that shows that RHAMM may not be constitutively expressed by all blast (Tzankov *et al.* 2011). In MM was found high expression of its three different variants (full-length RHAMM, RHAMM-48 and RHAMM-147) (Crainie *et al.* 1999).

The overexpression of *RHAMM* mRNA and protein has been associated to a poor outcome and increased peripheral metastasis in breast cancer (Wang *et al.* 1998), then suggested as a susceptible gene. The homozygous variation is associated to the early onset of the breast cancer (Pujana *et al.* 2007). Recently, the expression of RHAMM has been reported to be a negative prognostic factor in AML as well (Tzankov *et al.* 2011).

RHAMM was identified as one of the most promising LAAs in AML (Greiner *et al.* 2002, Schmitt *et al.* 2008). The nonamer ILS LEL MKL (designated R3), position 165-173, is the most immunogenic epitope (Greiner *et al.* 2005) and it can be naturally processed and presented in an HLA-A2 restricted manner. RHAMM-R3 elicits both humoral and cellular responses in patients with leukemias but not in healthy donors or patients with autoimmune diseases (Greiner *et al.* 2002, Greiner *et al.* 2003, Schmitt *et al.* 2008). Therefore, the presence of anti-RHAMM antibodies is not related to autoimmunity but to specific recognition of RHAMM by B cells. Moreover, RHAMM-specific CTLs are able to lyse autologous $RHAMM^{+}$ blasts and the deficient-in-transporter-associated-with-antigen processing (TAP) T2 cells pulsed with RHAMM-R3 peptide (Li *et al.* 2005, Greiner *et al.* 2005).

Clinical trial using RHAMM-R3 in AML, MDS, MM and CLL developed specific immune responses. Functionally active RHAMM-R3 CTLs were detected by tetramer staining in 70% of patients (Schmitt *et al.* 2008, Greiner *et al.* 2010, Giannopoulos *et al.* 2010). Peptide vaccination with RHAMM-R3 was safe and effective to mount immune responses in leukemia patients.

1.3 Tumor suppressor p53

The tumor suppressor *p53* gene is a transcription factor mapped to the short arm of the chromosome 17 (17p13.1). It is activated in response to stress like heat shock, DNA damage and oncogene activation. p53 regulates a broad group of genes involved in cell-cycle, apoptosis, metabolism, DNA repair and stem cell activity (Godar and Weinberg 2008, Sohr and Engeland 2008). Mutant p53 can no longer bind DNA in an effective way; therefore, its tumor suppressor activity may be abrogated. Inactivated, cytoplasmic mislocated or mutated p53 has been reported in most of the solid tumors including gliomas, melanoma, lung, breast, liver and colon cancer (Stretch *et al.* 1991, Clark *et al.* 2007, Sohr and Engeland 2008, Prokocimer and Peller 2011). Usually, hematological malignancies have no mutations in the p53 gene. In AML and MDS, p53 is mutated in 17% and 10%; respectively (Krug *et al.* 2002). It is rarely mutated in *de novo* AML (~15%), but it is mutated in 78% of AML patients with complex karyotype, usually due to the loss of the 17p chromosome (57% - 69% of the cases). Only 3.1% of patients without deletion of the chromosome 17p had p53 mutations (Lai *et al.* 1995, Prokocimer and Peller 2011). Abrogation of the p53 pathway has been also reported in AML. Furthermore, p53 may be inactivated by cytoplasmic sequestration of the protein in AML (Prokocimer and Peller 2011), just as reported in solid tumors. Chemotherapy-related AML and MDS are also associated with alterations in the p53 pathway. Inactivation of p53 has been reported in CML. During the blast crisis phase 21% had deletions and 19% had mutations of p53 (Krug *et al.* 2002). Moreover, p53 is overexpressed in ALL and B-CLL (Prokocimer *et al.* 1986).

1.4 Aim of the project

Previous studies by our group (Schmitt *et al.* 2008) and others (Mailänder *et al.* 2004, Oka *et al.* 2004, Keilholz *et al.* 2009, Maslak *et al.* 2010) demonstrated that the vaccination with peptides derived from RHAMM and WT1 can elicit T cell responses and that those may be associated with a good clinical outcome of the patients. Here we aimed to determine the expression of *RHAMM* and *WT1* and the spontaneous presence of specific T cells reacting against these LAAs in patients with hematological malignancies. We investigated the response of patients before and after allogeneic SCT and/or conventional chemotherapy. Furthermore we correlated the expression of *RHAMM* and *WT1* with the tumor suppressor *p53*.

We hypothesized that *RHAMM* may also serve as a marker to detected MRD as we confirmed it for WT1, a MRD marker previously described by others (Cilloni and Saglio 2004, Cilloni *et al.* 2008, Cilloni *et al.* 2009, Nowakowska-Kopera *et al.* 2009).

Moreover, we investigated the expression of cytokines in patients before and after treatment, and correlated them with their clinical status.

The specific objectives of this study were *i*) to determine and correlate the expression of *RHAMM*, *WT1* and *p53* in patients with hematological malignancies before and after treatment, *ii*) to correlate the expression of *RHAMM*, *WT1* and *p53* with clinical parameters, *iii*) to determine the immune response before and after treatment, *iv*) to determine the expression of the cytokines before and after treatment.

2 MATERIALS AND METHODS

2.1 Patient samples and healthy donor samples

We obtained 256 peripheral blood samples and 126 bone marrow aspirations from 69 patients suffering from leukemia. Serial peripheral blood and bone marrow samples were collected at diagnosis, after chemotherapy and/or after allogeneic SCT with immunosuppression, in CR or during maintenance therapy at sequential time intervals during following-up and at relapse.

Peripheral blood and bone marrow samples were diluted in 1x PBS (from DPBS 10x, Invitrogen Darmstadt, Germany). Peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs) were separated using Ficoll-Biocoll separating solution (Biochrom AG, Berlin, Germany) density gradient centrifugation and cryopreserved in RPMI-1640 (Biochrom) complete medium supplemented with 20% heat-inactivated human AB serum (DRK Blutspendedienst, Mannheim, Germany) and 10% DMSO (Sigma-Aldrich, Steinheim, Germany) according to standard protocols.

Peripheral blood samples were collected from 10 healthy donors and used as controls to define the normal expression of the target genes and T cell immune

responses. A general scheme of the method is illustrated in Figure 1. Bone marrow RNA was kindly provided by Prof. Dr. med. Anthony D. Ho, University of Heidelberg, Germany.

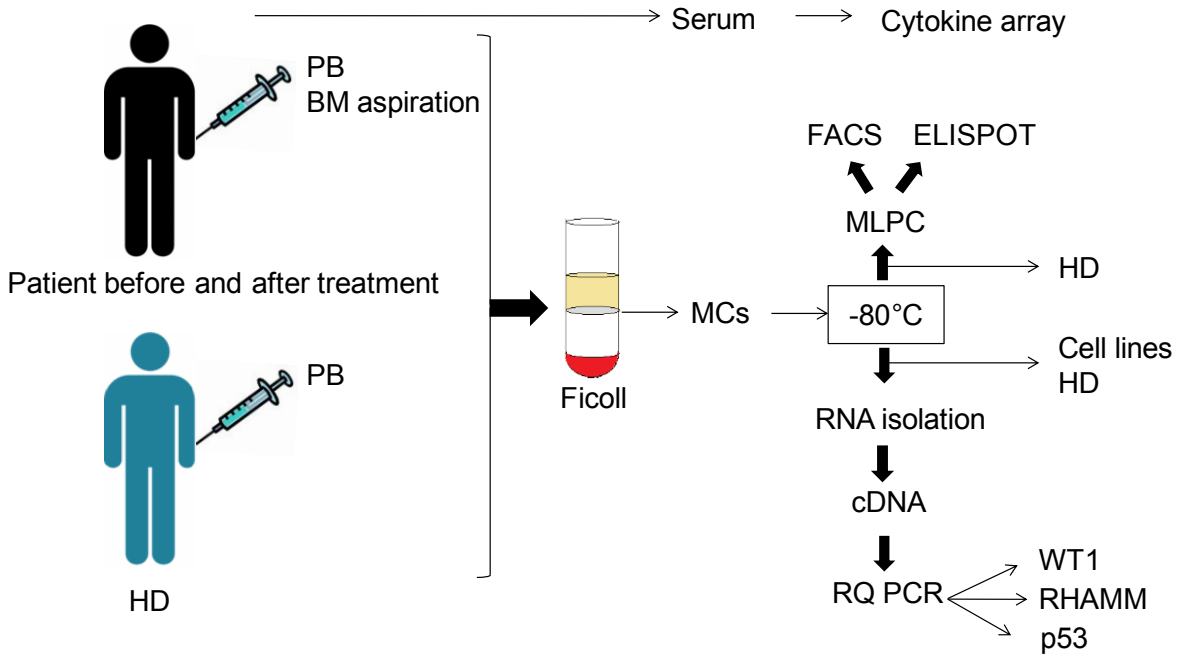

Figure 1. General scheme of the method. Peripheral blood (PB) and bone marrow (BM) aspirations were collected from patients before and after treatment as well as peripheral blood from healthy donors (HD). Mononuclear cells (MCs) were separated using Ficoll density gradient centrifugation and cryopreserved immediately at -80°C and afterwards in liquid nitrogen. Samples were harvest for either MLPC or RQ PCR. Healthy donor samples were used as negative controls, whereas cell lines such as K562 were used as positive controls. Sera were collected from patients before and after treatment to analyse the expression of cytokines through a cytokine array.

2.2 Cell lines

K562 and T2 cell lines were obtained from University of Ulm, Germany. OCI-AML 2 and OCI-AML 3 were obtained from Prof B. M. Pützer, University of Rostock. Nalm-6 and REH were obtained from PD Dr. M. Linnebacher, University of Rostock. All cell

lines were mycoplasm-free as detected by conventional RT-PCR (forw 5' GGCGAATGGGTGAGTAACACG 3'; rev CGGATAACGCTTGCGACCTATG 3'). Table 4 resumes the origin of the cell lines.

Cell line	Origin
K-562	Cell line derived from a 53-year-old CML patient in blast crisis. Cells carry the Philadelphia chromosome with a b3-a2 fusion gene
OCI-AML 2	Established from the peripheral blood of a 65-year-old man with AML, M4 at diagnosis in 1986
OCI-AML 3	Established from the peripheral blood of a 57-year-old man with AML, M4 at diagnosis in 1987
Nalm-6	Established from the peripheral blood of a 19-year-old man with ALL in relapse
REH	Established from the peripheral blood of a 15-year-old North African girl with ALL at first relapse
T2	Established by fusion of the B-lymphoblastoid cell line (LCL) with an 8-azaguanine and ouabain-resistant variant of the T-LCL CEM

Table 4. Origin of the cell lines according to the DSMZ. The cell lines K-562, OCI-AML 2, OCI-AML 3, Nalm-6, REH were used as positive controls for the measurement of target genes, while T2 was used as target cell in ELISPOT assays.

2.3 Real time PCR

2.3.1 RNA isolation

Cells were thawed once and washed with 1x PBS. RNA was isolated from a minimum of 2 x10^6 cells using RNeasy plus mini kit (QIAGEN, Düsseldorf, Germany).

RNA concentration and purity were measured by a Nano Drop at 260 nm. Only samples with a ratio ~2.0 and a concentration higher than 40 ng/µl were reversely transcribed into cDNA. Remained RNA was kept at -20°C. RNA samples were not thawed more than three times. To prevent any contamination with RNases in the samples, pipettes were irradiated for one hour before any procedure and surfaces were cleaned with RNase Zap (Ambion, Applied Biosystems, California, USA).

2.3.2 Absolute quantification of *WT1*

WT1 absolute copy numbers were measured with the WT1 profile *Quant* Kit (ELN) (IPSOGEN, Marseille, France) according to manufacturer's instructions. Briefly, 400 ng - 1000 ng of RNA was reversely transcribed into cDNA using the iScript cDNA synthesis kit (BioRad, Munich, Germany). The reverse transcription product (RT) was diluted with 30 µl of molecular–biology water (Sigma-Aldrich). *ABL* expression was used as the endogenous cDNA quantity control for all samples. Individual standards of *WT1* (10^1, 10^2, 10^3, 10^5, 10^6/5 µl) and *ABL* (10^3, 10^4, 10^5/5 µl) were run with each sample to calculate the appropriated standard curve. Ratios are expressed as *WT1* copy numbers/*ABL* 10^4 copy numbers. Reactions were performed by duplicate in an AbiPrism 7900 platform (Applied Biosystems) using standard conditions with 50 cycles of amplification in 25 µl of volume. TaqMan®2xPCR Master Mix (Applied Biosystems) was used as a buffer according to manufacturer's recommendations. Absolute copy numbers of *WT1* were considered only if the copy numbers of *ABL* were higher than 2000.

2.3.3 Absolute quantification of *RHAMM* and *p53*

Five hundred nanograms of RNA were reversely transcribed into cDNA using the iScript cDNA synthesis kit. RTs were diluted with 70 µl of molecular–biology water. Nine µl were used per well. Primers/probes (TaqMan® Gene Expression Assays, Invitrogen) were diluted in TaqMan®2xPCR Master Mix according to manufacturer's instructions.

We developed standard curves of *RHAMM*, *p53* and *ABL* in order to quantify absolute copy numbers of each gene. *ABL* expression was used as the endogenous cDNA quantity control for all samples. We chose *ABL* to normalize the copy numbers of the target genes for two reasons, *i*) *ABL* gene did not differ significantly between normal and leukemic samples at diagnosis (Beillard *et al.* 2003), and *ii*) to compare the expression of *RHAMM* and *p53* with *WT1*. Vectors were kindly provided by Prof. B. M. Pützer, University of Rostock. Copy numbers were calculated by www.endmemo.com. Four standard detections diluted 1-log were used per gene. Individual standards were run with each sample. Reactions were performed using ABI PRISM 7900 sequence detection system (Applied Biosystems) in duplicate using standard conditions with 40 cycles of amplification in 20 µl of volume.

2.4 Mixed lymphocyte peptide culture (MLPC)

PBMCs and BMMCs before and after transplantation were thawed once and washed with RPMI containing 1% penicillin/streptomycin (Invitrogen Gibco, Grand Island,

USA) and 1% L-Glutamine (Biochrom AG). Cells were washed and resuspended in magnetic-activated cell sorting (MACS) buffer (AutoMACS rinsing solution, Miltenyi Biotec, Bergisch-Gladbach, Germany) containing bovine serum albumin (BSA, Serva Electrophoresis GmbH, Heidelberg, Germany). $CD8^+$ specific T cells were separated by magnetic beads (Miltenyi) through a MACS column (Miltenyi). More than 95% purity was reached in the $CD8^+$ fraction. $CD8^-$ APCs were irradiated with 30 Gy and loaded with test or control peptides (20 µg/ml) or cultured with medium alone (no-peptide) for 2 hrs at 37°C with 5% CO_2. $CD8^-$ APCs were stabilized with 2.5 µg/ml of β-2 microglobulin (Sigma-Aldrich). Test peptides included RHAMM (ILS LEL MKL) and WT1 (RMF PNA PYL) derived peptide, and control peptides included the derived pp65 peptide from the cytomegalovirus (CMVpp65, NLV PMV ATV) or the influenza matrix protein (IMP, GIL GFV FTL) based on the pre-exposure of healthy donors and patients to CMV (see clinical status section). $CD8^+$ ($5x10^5$) and $CD8^-$ ($20x10^5$) were placed in 24-well plates in a ratio of 1:4. After overnight incubation at 37°C with 5% CO_2, the MLPC was supplemented with 10 U/mL IL-2 (Sigma Aldrich) and 20ng/mL IL-7 (Miltenyi) on day 1. CTLs were harvested on day seven for enzyme linked immunospot (ELISPOT) assay and/or flow cytometry analysis if sufficient number of $CD8^+$ cells were collected.

2.4.1 Mini-MLPC

The MLPC approach was modified into a mini-MLPC in case that not enough $CD8^+$ cells were obtained after MACS separation. Mini-MLPCs were performed in round-

bottomed 96-well microtiter plates in RPMI-1640 culture medium supplemented with 10% heat-inactivated human AB serum, 10 U/mL IL-2 and 20 ng/mL IL-7. The ratio was maintained as in the MLPC ($1x10^4$ $CD8^+$ and $4x10^4$ $CD8^-$, 1:4). Number of cells per well was based on worked by Distler (2007). The proliferation was comparable to that occurring in a conventional MLPC.

2.5 Enzyme-linked immunospot (ELISPOT)

Functional evaluation of antigen-specific $CD8^+$ T cells was determined by the release of interferon gamma (IFNγ) and granzyme B in different ELISPOT assays.

2.5.1 IFN-γ ELISPOT

ELISPOT plates (MultiScreen IP 96-well plates, Millipore, Massachusetts, USA) were coated with anti–human IFN-γ (Mabtech, Nacka Strand, Sweden) re-suspended in coating buffer (see Supplement 1) and incubated overnight at 4°C. Plates were washed with 1x PBS and blocked with 1x PBS containing 10% AB serum for 2 hrs at RT. T2 cells were used as targets. T2 cells were loaded with or without respective peptides or cultured in medium alone (no peptide) and stabilized with β-2 microglobulin for 2 hrs at 37°C with 5% CO_2. They were washed in standard media to remove excess peptide. To measure the antigen-specific $CD8^+$ T cell responses, viable $1x10^4$ $CD8^+$ and $4x10^4$ T2 cells/well were incubated overnight at 37°C with 5%

CO_2 in triplicates. After washing with 1x PBS, followed by 1x PBS containing 0.05% tween 20 (Sigma-Aldrich), biotin-linked secondary antibody was incubated for 2 hrs at RT and washed with 1x PBS 0.05% tween 20. Streptavidin-ALP-linked antibody was incubated for 2 hrs at RT and washed with 1x PBS 0.05% Tween 20 and subsequently with substrate buffer (see Supplement 1). The reaction was developed with BCIP/NBT Liquid Substrate System (Sigma-Aldrich) for 3-10 min until spots were detected. Reactions were stopped with distilled water (Fresenius Kabi Deutschland GmbH, Hamburg, Germany). Specific T cell responses were considered positive if there were a minimum of 10 spots (after subtracting the number of spots in unstimulated control). Spots were counted using an automated ELISPOT reader (CTL, Bonn, Germany). Data were analysed with ELISPOT software (CTL).

2.5.2 Granzyme B ELISPOT

ELISPOT plates (MultiScreen IP 96-well plates, Millipore) were coated with anti–human granzyme B (BD Biosciences, Heidelberg, Germany) re-suspended in 1x PBS and incubated overnight at 4°C. Plates were washed with RPMI-1640 containing 10% FBS and blocked the same medium for 2 hrs at RT. Pulsed T2 cells, as described previously, were co-cultured with respective specific T cells at 37°C with 5% CO_2 overnight by triplicates. After washing with distilled water, and subsequently with 1x PBS 0.05% tween 20, biotin-linked secondary antibody was incubated for 2 hrs at RT and washed with 1x PBS 0.05% tween 20. Streptavidin-

HRP linked antibody was incubated for 1 hr at RT and wash with 1x PBS 0.05% Tween 20 and subsequently with 1x PBS. The reaction was developed with AEC chromogen (BD) for 5-60 min until spots were detected. Reactions were stopped with distilled water. Specific T cell responses were considered as previously explained. Spots were counted and data were analyzed as previously described (Section 2.5.1).

2.6 Flow cytometry

The frequency of antigen-specific T cells was determined by flow cytometry. Briefly, cells were harvest after one week stimulation in MLPC or mini-MLPC and washed with 1x PBS containing 1% BSA. Lymphocytes were stained with Antigen-specific tetramers for 40 min at RT in the darkness. Conjugated antibodies to CD3 and CD8 (BD Biosciences) were added and incubated for 20 min at 4°C. Fluorescein isothiocyanate (FITC), peridinin-chlorophyll protein (PerCP) and phycoerythrin (PE) were used as fluorophores. Cells were washed with 1x PBS to remove any unbound antibody. A minimum of $2x10^4$ cells were acquired. Flow cytometry was performed on a Calibur cytometer (BD Biosciences) using the same settings for each experiment. Appropriated isotype control was analysed with each experiment. Data were analyzed using flow cytometry analysis software (FlowJo, Tree Star, Inc, USA). The frequency of tetramer $CD8^+$ T cells was considered positive if it was 2-fold or higher than the frequencies of irrelevant peptide-tetramer.

2.7 Cytokine array

Serum samples were collected before and after treatment, either allogeneic SCT or chemotherapy, and stored at -20°C until use. All of them were thawed only once. Cytokines (C5a, CD40-L, G-CSF, GM-CSF, CXCL1, CCL1, sICAM-1, IFNγ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-17p70, IL-13, IL-16, IL-17, IL-17E, IL-23, IL-27, IL-32α, CXCL10, CXCL11, MCP-1, MIF, MIP-1β, serpin E1, RANTES, CXCL12, TNF-α, sTREM-1) were measured by duplicate with the Proteome profiler^{TM} kit (R&D Systems, Abingdon, UK) according to manufacturers' instructions. Briefly, membranes containing capture antibodies were incubated with provided blocking buffer for 1 hr at RT. Sera were incubated with a cytokine array cocktail at RT. Membranes were incubated with the sera/antibody at 4°C overnight for optimal sensitivity. After washing three times with provided washing buffer, membranes were incubated with streptavidin-HRP for 30 min at RT and washed again. All of the membranes were incubated at the same time with chemiluminiscent detection substrate (Amersham, GE, Uppsala, Sweden) for 3 min at RT. Membranes were exposed to an X-ray (Kodak BioMax Light 1, Sigma-Aldrich) for 10 min. The film was developed in an automatic processor according to conventional protocols. Signals were quantified by the pixel density using the image processing-program ImageJ. Average background signal was subtracted from the average of each testing detected-cytokine. Corresponding signals of different arrays were compared to determine relative changes in cytokine levels. Cytokine expression during relapse and CR was considered to be different in a single patient when the pixel density was bigger than 10,000 as an arbitrary number.

2.8 Clinical status of the patients

The clinical status of patients was obtained from the “Arztbriefsystem” of the University of Rostock. Clinical features of the patients such as chimerism analysis, cytogenetics, HLA, CMV status, therapy were determined by the Department of Internal Medicine III, University of Rostock. The *FLT3* status was kindly provided by the Department of Hematology/Oncology at the University of Greifswald, Greifswald, Germany.

2.9 Criteria for analysis of the data

In order to make the data as uniform as possible for further statistical analysis, we considered the highest value before treatment, either transplantation or first induction chemotherapy. After the day of treatment we grouped the following-up every three months and considered the highest value of each following-up group.

To analyze if patients expressed different copy numbers according to gender or karyotype, we considered all of the patients’ data before treatment.

We considered only those patients positive for the target gene, either *RHAMM* or *WT1* for further analysis with the genes.

2.10 Statistical analysis

Statistical analyses were performed using Stat Graphics Plus 5. Statistical analyses showed that data were non-parametric. Therefore, Mann Whitney U test was used to compare the expression of the LAAs at different stages of the disease of patients, due to the lack of data in all of the following-ups. Correlations were obtained with Spearman test. Survival analyses were obtained by Kaplan-Meier test (Graph Pad). Statistical significance was considered if the p-value was <0.05.

3 RESULTS

3.1 Patient features

We screened 69 patients in a prospective study of 2.5 years (55 AML/MDS, 6 ALL, 6 CLL and 2 CML). Thirty-three patients received allogeneic SCT, whereas 36 received only chemotherapy under conventional protocols (Tables 5 and 6). Our cohort of patients maintained a ratio of almost 1:1 between men (n=32) and women (n=37). We noticed that more men had a complex karyotype when compared to women (8 and 3; respectively). At the time of the enrollment, 26 patients were diagnosed with AML (female=15, men=11). Complex karyotype was confirmed in six men and in two women. Normal karyotype was prevalent in women (n=15), compared to men (n=9). There was no significant difference between the age of men and women (p=0.5) at the time of diagnosis.

AML cases were classified according to the FAB criteria and characterized at the cytogenetic level. Main clinical and biological features are summarized in Tables 5 and 6. CR was defined according to standard criteria.

The mean following-up time of the patients was 269 days (median: 216 days). Thirteen patients had a deceased related to the disease and five patients died due to causes non-related to the leukemia (Tables 5 and 6).

The mean overall survival (OS) in all of the patients was 274 days (median: 276 days). AML/MDS patients had a mean OS of 265 days (median: 269), whereas the mean OS of ALL, CLL and CML patients was 247, 452 and 163 days; respectively (median: 226, 479 and 163 days; respectively) until the end of the study (Figure 2). $WT1^+$ patients had a lower OS compared to $WT1^-$ patients (Figure 3).

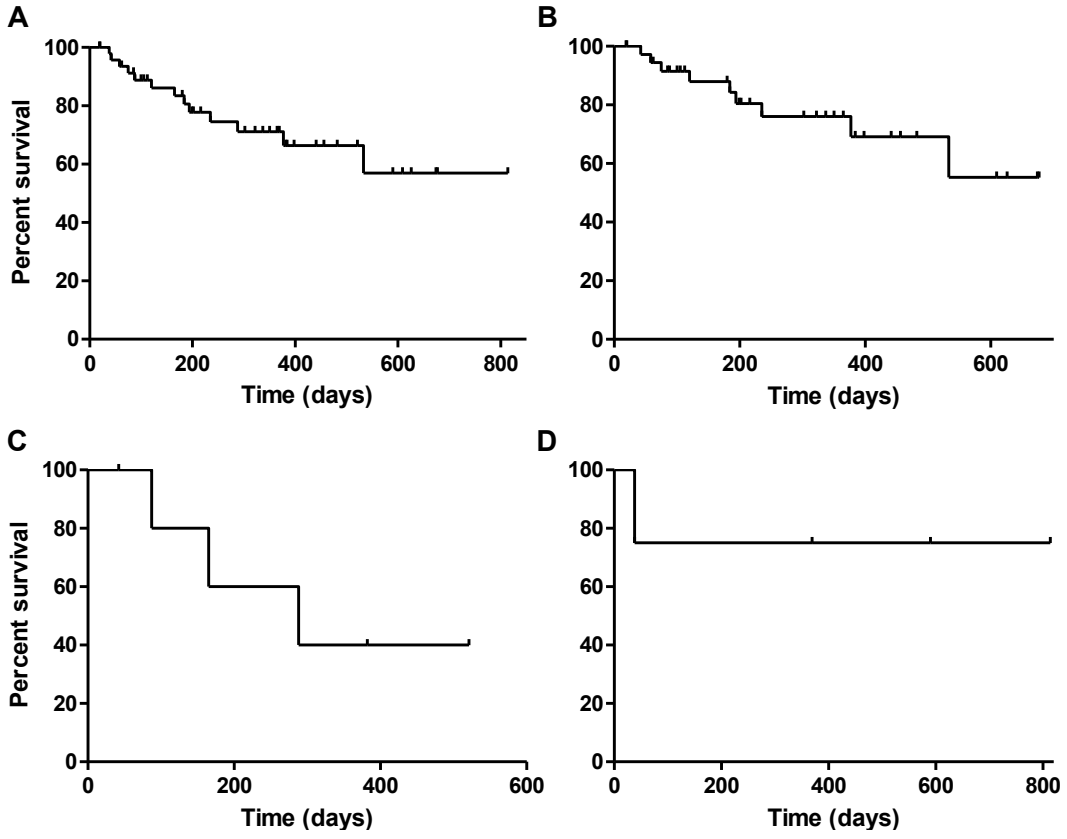

Figure 2. Survival of the 69 patients included in this study. A. Overall survival of all of the patients, **B.** Survival percentage of AML/MDS patients, **C**. Survival percentage of ALL patients, **D**. Survival percentage of CLL patients. The number of patients with CML was too small to be analyzed by Kaplan-Meier tests.

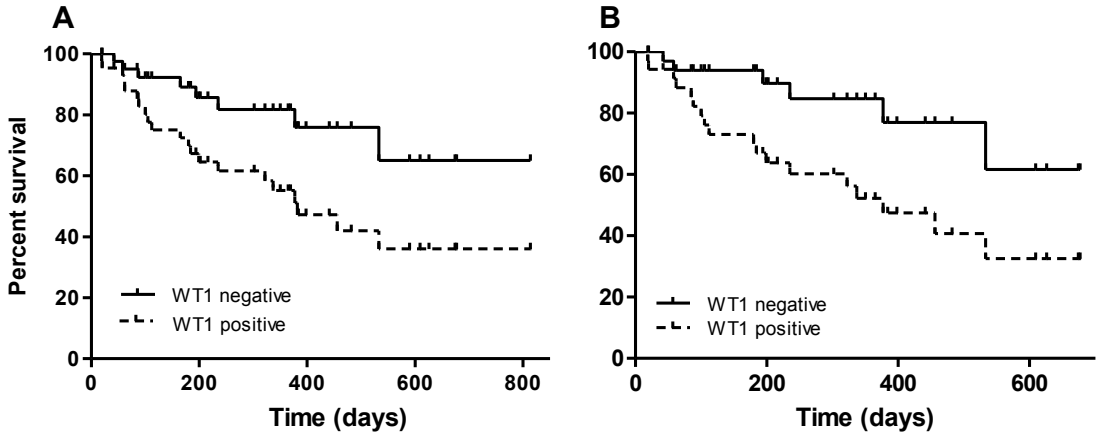

Figure 3. *WT1*$^{+}$ patients (n=22) had a lower OS than *WT1*$^{-}$ patients (n=27). WT1 status was considered before treatment. **A**. Survival percentage of all of the patients (p=0.01), **B**. Survival percentage of AML/MDS patients (p=0.01).

Patient number	Disease	Classification	Sex	Age	Karyotype	Cytogenetics	HLA type	*FLT3-ITD*	Conditioning	Clinical status*	Survival	WT1	RHAMM
1	MDS	RAEB-II	F	60	Normal	46,XX	A3, A24	NR	Bu Flu	CR	Alive	Neg	Neg
2	sAML	NA	M	57	Complex	del5q7q12p. Complex rearrangement of chromosomes 2, 4, 8, 21	A2, A25	Neg	Bu Flu	CR	Alive	Neg	Pos
3	AML	M1	F	56	NR	NR	A2	Neg	Bu Flu	CR	Alive	Neg	Only BM
4	AML	M0	F	47	Normal	46,XX	A1, A2	Neg	FLAMSA	Rel	Dead (leukemia)	Pos	Only BM
5	AML	M5	M	50	Normal	46,XY	A2	Neg	Treo Flu	CR	Dead (leukemia)	Pos	Pos
6	AML	M4	F	53	Normal	46,XX	A2	Pos	Treo Flu	RD	Alive	Pos	Neg
7	sAML	NA	F	42	Normal	46,XX	A3, A26	NR	Treo Flu	CR	Alive	Pos	Only BM
8	AML	M2	F	57	Complex	t(8;21),-X,del(9q)	A3, A31	Neg	Treo Flu	CR	Dead (leukemia)	Neg	Neg
9	AML	M5	F	64	Aberrant	47,XX,+8,del(11)(q23)	A11, A30	Neg	FLAMSA	CR	Alive	Pos	Pos
10	sAML	NA	F	54	Aberrant	Isochromosome 17(q10)	A1, A2	NR	FLAMSA	PR	Dead (encephalitis)	Neg	Pos
11	MDS/MPS	NA	F	67	Normal	46,XX	A1	NR	Treo Flu	CR	Alive	Pos	Neg
12	AML	M4	F	27	Aberrant	t(6;9)(p21-22; q34)	A23, A66	Neg	FLAMSA	CR	Alive	Pos	Pos
13	MDS	RAEB-II	M	57	Normal	46,XY	A1, A2	NR	Treo Flu	Rel	Alive	NGR	NGR
14	AML	M5	M	42	Aberrant	t(10;11)(p1,2;q23)	A3	NR	Treo Flu	CR	Dead (GvHD)	Neg	Pos
15	sAML	NA	F	44	Normal	46,XX	A1, A24	Neg	FLAMSA	CR	Alive	Neg	Pos
16	AML	M2	M	59	Aberrant	t (8;21),del(9q)	A2, A24	Neg	Treo Flu	CR	Alive	Neg	Pos
17	AML	M4	M	56	Normal	46,XY	A2, A28	Neg	Bu Flu	CR	Alive	Neg	Pos
18	AML	M7	M	45	Aberrant	47,XY,t(3;11)(p21;q23),+8	A1, A11	NR	NR	RD	Alive	Pos	Pos
19	MDS	RAEB-II	F	48	Aberrant	45,XX,-7	A2	NR	FLAMSA	RD	Dead (neumonia)	Pos	Neg
20	MPS	NA	F	59	Normal	46,XX	A3, A24	NR	Treo Flu	CR	Alive	NGR	Only BM
21	MDS	RAEB-II	M	70	Complex	42~46,XY,del(7)(p10),+9,der(20), t(7;20)(p21;p13)	A2	NR	Treo Flu	CR	Alive	Neg	Only BM
22	AML	M4	F	48	Normal	46,XX	A2, A29	Neg	FLAMSA	Rel	Alive	Pos	Neg
23	B-ALL	NA	M	64	Complex	45X-Y, inv(3)(p24q36),del(7)(q11), del(7)(q21q31),add(8)(p),del(13)(q14q22) , del(14), trisomy 9q34	A2, A24	NA	Flu TBI (8 Gy)	CR	Alive	Pos	Pos
24	T-ALL	NA	M	43	NR	NR	A1, A2	NA	Eto TBI (12 Gy)	CR	Alive	NGR	NGR
25	ALL	NA	F	26	Normal	46,XY	A3, A11	NA	Cy TBI (12 Gy)	CR	Dead (leukemia)	Neg	Pos
26	B-ALL	NA	M	42	Complex	48,XY,+der(5),t(5;22),-22, +der(22),t(9;22)(q34;q11.2)	NR	NA	Eto TBI (12 Gy)	CR	Alive	Neg	Pos
27	ALL	NA	F	40	NR	NR	A2, A3	NA	Cy TBI (12 Gy)	CR	Dead (heart failure)	Neg	Pos
28	CML	NA	F	43	Aberrant	46,XX, t(9;22)(q34;q11.2)	A2, A32	NA	Bu Cy	CML-BC	Alive	NGR	NGR
29	CML	NA	F	62	NR	NR	A2, A3	NA	Treo Flu	CP	Alive	Pos	Only BM
30	B-CLL	NA	F	57	Complex	t(2;14)(p13;q32),del(11q22.3),t(14q32)	A1, A2	NA	Treo Flu	PR	Alive	NGR	Pos
31	B-CLL	NA	M	49	NR	NR	A3, A25	NA	Treo Flu	CR	Alive	Neg	Neg
32	B-CLL	NA	M	56	Aberrant	del(14q32)	A3, A25	NA	Flu TBI (2 Gy)	CR	Dead (Aspergillus)	Neg	Pos
33	CLL	NA	M	47	Aberrant	del(13)	A2	NA	Treo Flu	RD	Alive	Neg	Neg

Table 5 (Preceding page). Clinical features of all patients in this study that received an allogeneic SCT (n=33). All patients had an allogeneic transplant, with the exception of patient number 9, who received a syngeneic transplant. *WT1* and *RHAMM* positivity imply the expression of either gene at the beginning of the study, before they received any treatment. Patients were positive (pos) or negative (neg) in either peripheral blood or bone marrow for the transcripts *WT1* at any time before transplantation. Patients 5, 6, 7, 9, 11, 12 and 23 were enrolled in the study at diagnosis and they were $WT1^+$ positive at this moment. *RHAMM* positivity was based on the expression of the gene in the peripheral blood of patients. Transcripts in the bone marrow did not exceed the expression of *RHAMM* in healthy donors in the same compartment, therefore we could not make a statement of positivity. Transcripts were detected by RQ-PCR. *Clinical status is described immediately before transplantation. **Abbreviations.** add: additional material, origin unknown, Bu Cy: busulfan-cyclophosphamid, Bu Flu: busulfan-fludarabin, CML-BC: CML in blast crisis, CP: chronic phase, CR: complete remission, Cy: cyclophosphamid, del: deletion, der: derivative chromosome, Eto: etoposid, FLAMSA: fludarabin-cytarabin-amsacrin-4 Gy TBI-cyclophosphamid-mesna-ATG, GvHD: graft versus host disease, MPS: myeloproliferative syndromes, NA: not applicable, Neg: negative, NGR: the quality of the RNA was not good to obtain a result, NR: not reported, Pos: positive, PR: partial remission, RD: refractory disease, Rel: relapse, sAML: secondary AML, TBI: total body irradiation, Treo Flu: treosulfan-fludarabin, + : gain of a chromosome, - : loss of a chromosome

Table 6 (following page). Clinical features of the non-transplanted patients included in this study (n=36). Most patients received multiple therapies. Indicated here it is the therapy that patients received at the time of sampling (Treatment*). *The clinical status was considered when blood was taken for the first time. *RHAMM* and *WT1* status were considered as described in Table 5. **Abbreviations.** Allo-Tx: allogeneic transplantation, AraC: arabinoide C, ATRA: all-trans retinoic acid, CR: complete remission, Dx: diagnosis, FLAG: fludarabin arabinoide C G-CSF, MRD: minimal residual disease, NA: not applicable; Neg: negative, NGR: the quality of the RNA was not good to obtain a result, NR: not reported, Pos: positive, PR: partial remission, RBC: red blood cells, RD: refractory disease, RD: refractory disease, Rel: relapse, sAML: secondary AML, Vidaza: azacitidin

Patient number	Diagnosis	Classification	Sex	Age	Karyotype	Cytogenetics	HLA type	*FLT3-ITD*	Treatment*	Clinical status*	Blast % in BM	Survival	WT1	RHAMM
34	sAML	NA	M	74	Complex	41~44,XY,-Y,-4,-5,-7,-8, -9,-11,-13,-16,-19,-20,-21,-22	NR	NR	Vidaza	Dx	26%	Alive	Pos	Neg
35	AML	M4	F	56	Aberrant	46,XX,t(1;21)(q21;q22)	A1	Neg	Allo-Tx in 2008	CR	<5%	Alive	Neg	Pos
36	AML	M1	F	68	Aberrant	46,XX,t(21;22)(q22;q12)	A2, A24	Neg	Mitoxantrone, AraC	Dx	>30%	Alive	Pos	Pos
37	AML	M5	M	63	Normal	46,XY	A1, A3	Neg	Mitoxantrone, AraC	Rel	70-90%	Alive	Pos	Only BM
38	AML	M1	F	73	Aberrant	Monosomie 7	A1, A3	NR	Mitoxantrone, AraC	Dx	80-90%	Alive	Pos	Neg
39	AML	M4	M	61	Aberrant	46,XY,del(20)	A1, A11	Neg	Mitoxantrone, AraC	MRD	<5%	Alive	Neg	Only BM
40	AML	M5	M	25	Aberrant	47,XY,+21	NR	Pos	AraC, Daunorubicin, Midostaurin	Dx	80-90%	Alive	Pos	Pos
41	AML	M1	F	63	Normal	46,XX	NR	NR	Died after AraC pre-Phase	Dx	80-95%	Dead (leukemia)	Pos	Only BM
42	AML	M1	M	45	Complex	46,XY,t(8;21),-Y,del 9q	A1, A3	Neg	Cytarabine, Daunorubicin, Sorafenib	Dx	70%	Alive	Pos	Neg
43	sAML	NA	F	66	Normal	46,XX	A2, A32	NR	Mitoxantrone, Cytarabine	Dx	20%	Alive	Neg	Pos
44	AML	M5	F	67	Normal	46,XX	A24, A68	Neg	No treatment at the time of sampling	CR	<5%	Alive	Neg	Only BM
45	AML	M4	M	83	Complex	Complex	NR	NR	Vidaza	Dx	42%	Dead (leukemia)	Neg	Neg
46	AML	M4	M	71	Normal	46,XY	A2, A24	Neg	No treatment at the time of sampling	MRD	NR	Dead (leukemia)	NGR	Neg
47	AML	M5	F	61	Normal	46,XX	A2	Neg	Hydroxyurea followed by Mitoxantronee, Cytarabine	Dx	90%	Alive	Pos	Only BM
48	AML	M4	F	76	Normal	46,XX	A2	Neg	Mitoxantrone, Cytarabine	Dx	90%	Alive	Pos	Neg
49	MDS	RAEB-II	M	58	Normal	46,XY	A2, A28	Neg	Supportive care (RBC transfusion)	Dx	10%	Alive	Pos	Only BM
50	AML	M6	F	66	Normal	46,XX	A1, A3	Neg	No treatment at the time of sampling	CR	<5%	Alive	Neg	Pos
51	AML	M4	M	73	Aberrant	45,XY,-7	A2	Neg	Daunorubicin, Cytarabine	Dx	40%	Dead (leukemia)	Pos	Neg
52	AML	M4	M	63	Normal	46,XY	A26, A28	Pos	Clofarabin	Rel	NR	Alive	Pos	Pos
53	AML	M3	M	60	Aberrant	47,XY,+8,t(15;17)(q22;q21)	A2, A28	Neg	No treatment at the time of sampling	CR	<5%	Alive	Neg	Neg
54	sAML	NA	M	69	NR	NR	NR	NR	Mitoxantrone, Cytarabine	Dx	20%	Alive	Pos	Pos
55	AML	M4	M	75	Aberrant	47,XY,+8	NR	NR	Low dose of Mitoxantrone	PR	60-80%	Dead (leukemia)	Pos	Neg
56	MDS	RA	F	77	Aberrant	Monosomie 14	NR	NR	No treatment at the time of sampling	Dx	15%	Alive	Neg	Neg
57	AML	M1	M	21	Complex	49,XY,+8,del(13)(q12q14), +del(13)(q12q14)	A2	NR	Idarubicin, AraC	Dx	90%	Dead (leukemia)	Pos	Only BM
58	sAML	NA	F	78	NR	NR	A2	Neg	Low dose of AraC and Mitoxantrone, Litali	Dx	60%	Alive	Pos	Pos
59	sAML	NA	M	59	NR	NR	A2	Neg	Allo-Tx in 2007	CR	<5%	Alive	Neg	Only BM
60	AML	M4	F	68	NR	NR	NR	NR	Mitoxantrone-FLAG	CR	<5%	Alive	Pos	Pos
61	sAML	NA	F	82	NR	NR	A2	NR	Low dose of Mitoxantrone	Dx	89%	Alive	Pos	Pos
62	AML	M2	F	58	Complex	Complex	A2, A28	Neg	No treatment at the time of sampling	CR	<5%	Dead (leukemia)	Pos	Pos
63	AML	M0	M	66	Normal	46,XY	A1, A26	Neg	Allo-Tx in 2008	CR	<5%	Alive	Neg	Pos
64	AML	M4	F	57	Normal	46,XX	A1, A2	Neg	Cytarabine, Daunorubicin	Dx	40%	Alive	Neg	Neg
65	AML	M3	M	64	Aberrant	t(15;17)(q22;q21.1)	NR	Neg	ATRA, Idarubicin	Dx	90%	Alive	Pos	Pos
66	MDS	RAEB-II	M	67	Normal	46,XY	NR	NR	No treatment at the time of sampling	Dx	20%	Dead (leukemia)	NGR	NGR
67	B-ALL	NA	F	84	Normal	46,XX	NR	NA	Cyclophosphamide	Dx	94%	Dead (leukemia)	Pos	Pos
68	B-CLL	NA	F	46	NR	NR	NR	NA	Supportive care (RBC and platelet transfusion)	Progress	97%	Alive	NGR	Neg
69	B-CLL	NA	F	74	NR	NR	NR	NA	Rituximab mono	RD	82%	Alive	Neg	Only BM

3.2 Expression of LAAs in patients with leukemias before and after treatment

3.2.1 *WT1* transcripts

The median value of *WT1* copies/10^4 *ABL* copies in healthy donors was 3 (range 2-6) in peripheral blood and 71 (range 0-304) in bone marrow.

There was no difference in the expression of *WT1* in the transplanted group versus non-transplanted (p=0.5) at the time of diagnosis. Therefore, we analyzed the data all together. We found that 88% of all of the diagnosed patients were *WT1* positive. The median value of *WT1* copies/10^4 *ABL* copies was 1,666 (range 0 - $9.2x10^4$) in peripheral blood samples collected at the time of diagnosis (p=0.01 *vs.* healthy donors), and 3,377 (range 15 - $3.9x10^4$) in bone marrow (p=0.008 *vs.* healthy donors) (Figure 4). There was no significant difference in the expression of *WT1* between women and men (peripheral blood, p=0.56; bone marrow, p=0.65), neither in different karyotypes (peripheral blood: normal *vs.* complex, p=0.94; normal *vs.* aberrant, p=0.55; complex *vs.* aberrant, p=0.61; bone marrow: normal *vs.* complex, p=0.87; normal *vs.* aberrant, p=0.91; complex *vs.* aberrant, p=0.77). Furthermore, the whole cohort of patients at relapse was positive for *WT1*, whereas 33% of the patients in PR and 50% of the patients in RD were positive (Tables 5 and 6). All of the patients in CR (n=25), with the exception of patients number 60 and 62, showed *WT1* transcripts which were not higher than healthy donors. Patient No. 62 showed a clinical relapse two months after and died due to the leukemia (Table 6).

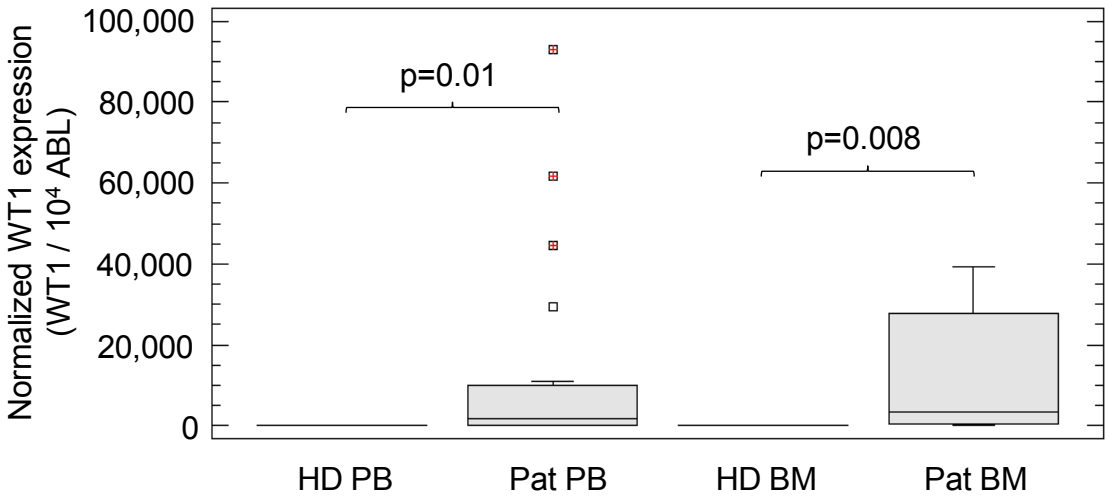

Figure 4. Normalized expression of *WT1* transcripts in all leukemias. *WT1* was measured in the peripheral blood of healthy donors (HD PB) and patients before treatment (Pat PB) as well as in the bone marrow of both (HD BM and Pat BM). The expression of *WT1* was higher in patients in both peripheral blood (p=0.01) and bone marrow (p=0.008).

Only patients that were positive for *WT1* before treatment were considered for the following-up analyses. We found that *WT1* was highly expressed in the peripheral blood of patients (p=0.05) and in bone marrow (p=0.001) before treatment when compared with the following-up months (Figure 5). We further analysed the expression of *WT1* in patients with a clinical good outcome that remained in CR and alive *versus* patients that die due to the leukemia. The transcripts of *WT1* were significantly different before treatment compared to the following-up in peripheral blood and bone marrow (Figure 6) in patients that survived. Figure 7 depicts an exceptional example of an AML patient with high copy numbers of *WT1* during relapse. After receiving allogeneic SCT, *WT1* transcripts diminished dramatically and remained as low as in healthy donors in both compartments, peripheral blood and

bone marrow. This low *WT1* expression is associated with the clinical CR of the patient.

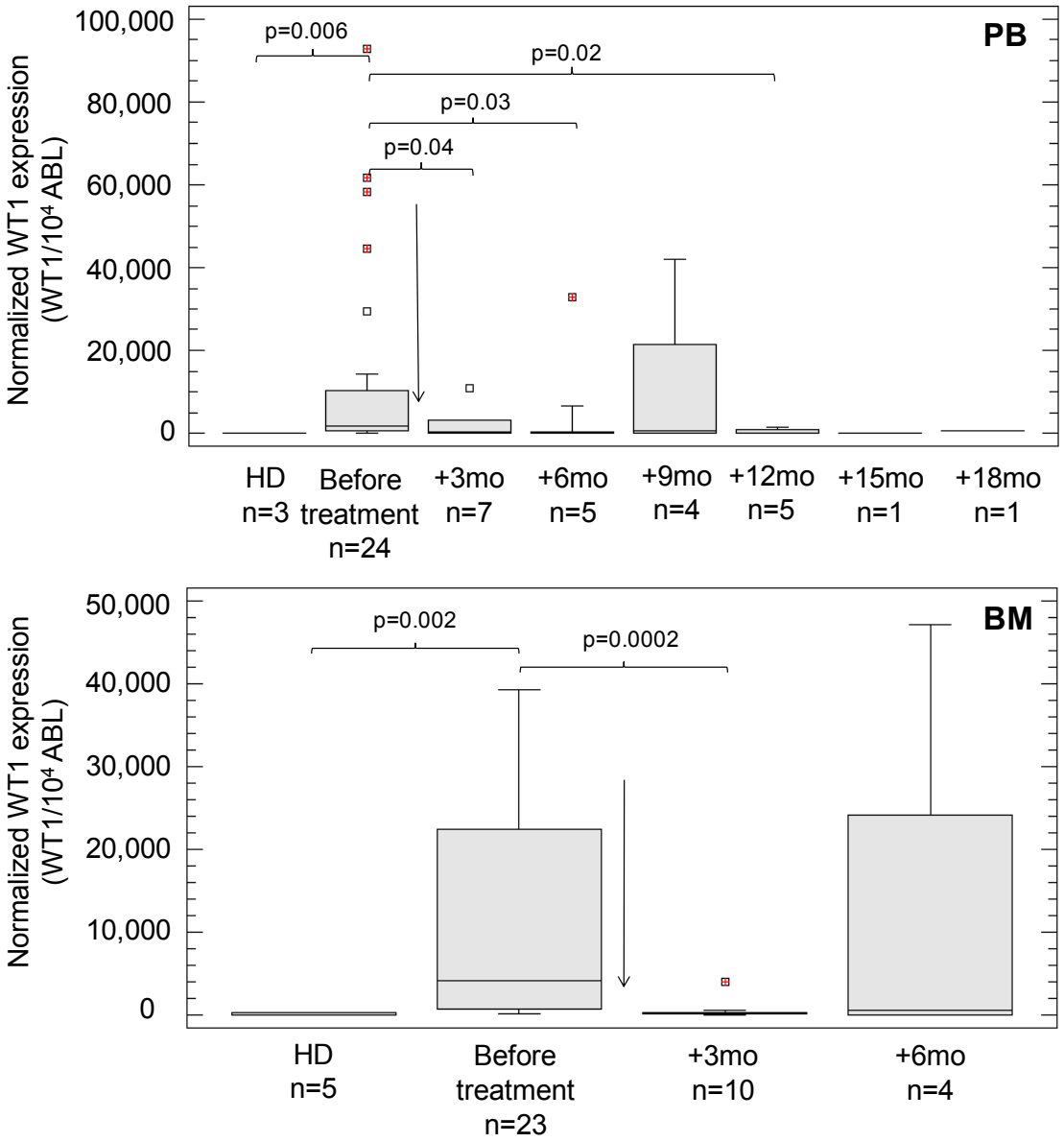

Figure 5. Normalized expression of *WT1* in patients. Expression of *WT1* in the peripheral blood (upper panel) and bone marrow of patients was higher before treatment compared to the expression after (depicted by an arrow). However after 9 months (mo) the expression of *WT1* was comparable to before treatment in the peripheral blood, as well as 6 months later in the bone marrow. n refers the number of samples that were obtained at a particular following-up.

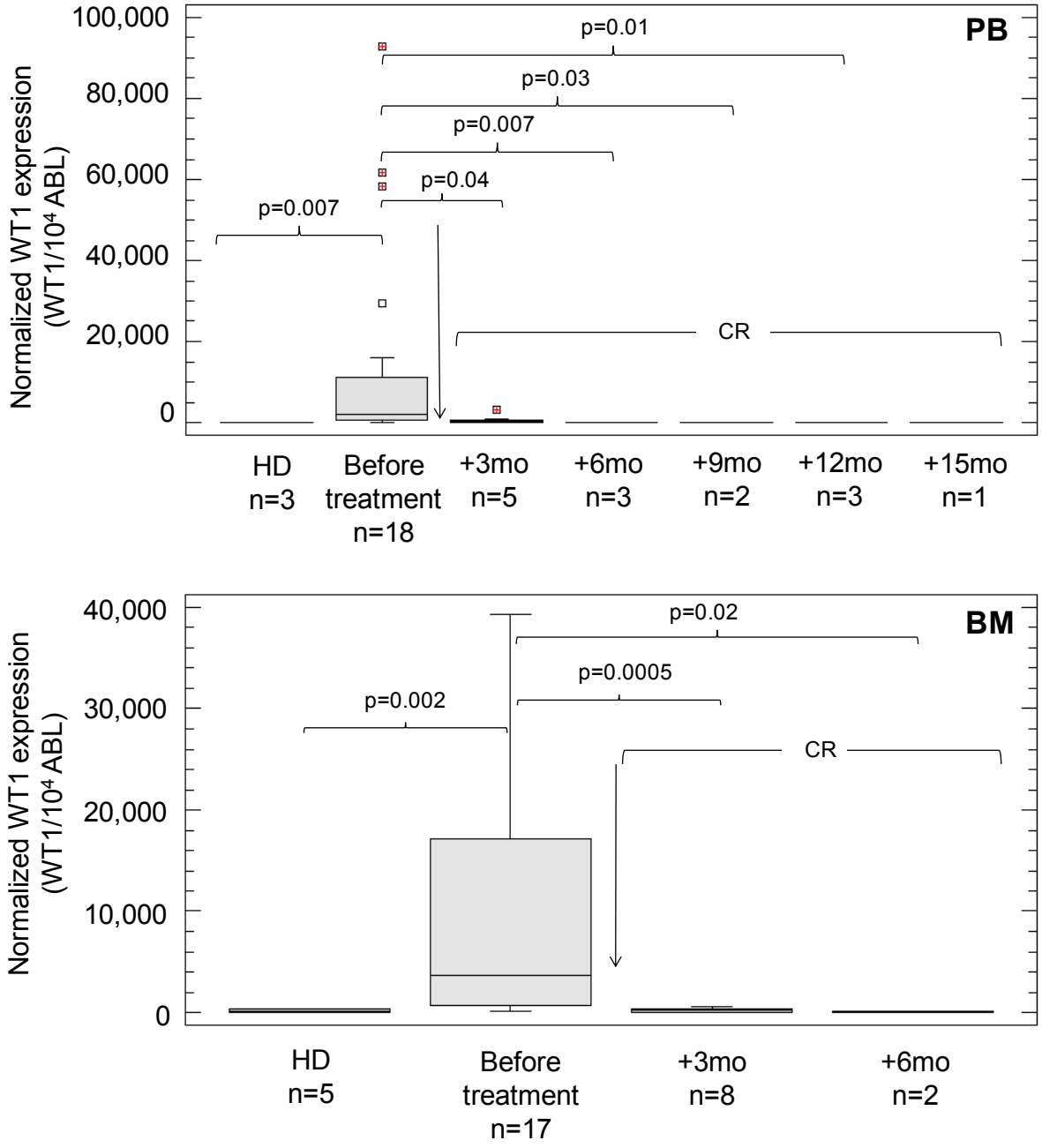

Figure 6. Normalized expression of *WT1* transcripts in patients with a good clinical outcome. Patients that remained in CR had low expression of *WT1* in peripheral blood (upper panel) and bone marrow for up to 15 months (mo) after treatment (depicted by an arrow). All of the patients were alive at the end of the study. n refers the number of samples that were obtained at a particular following-up.

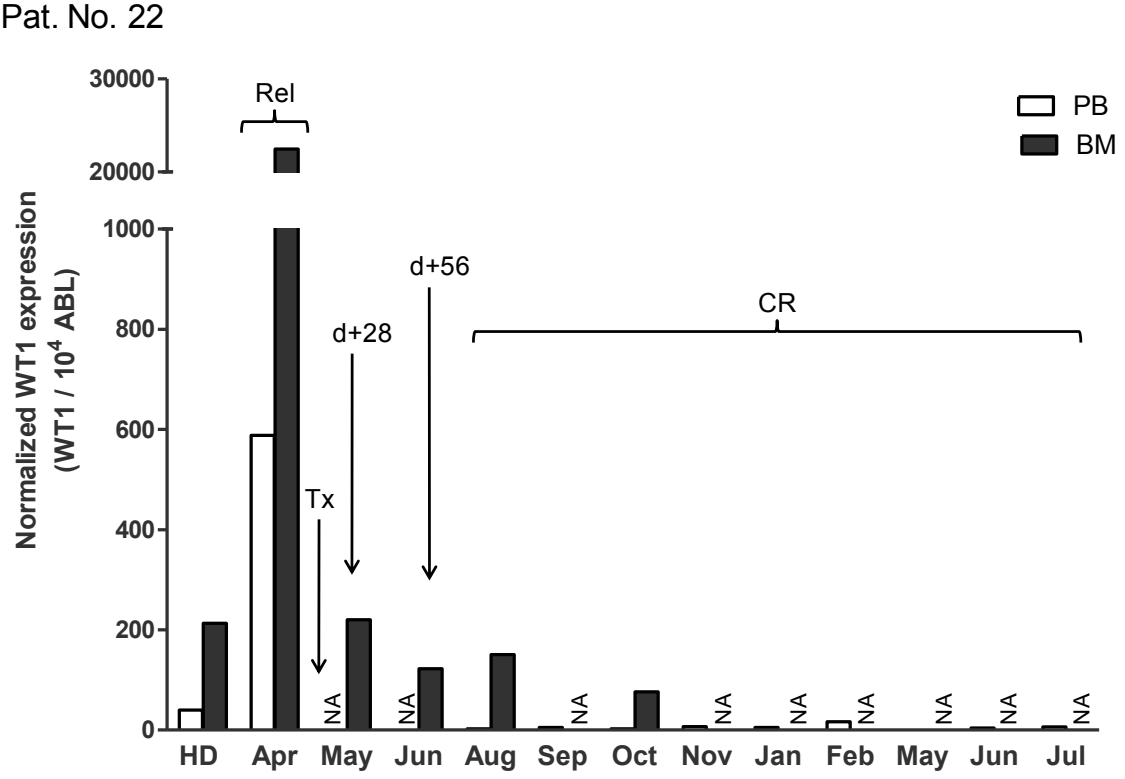

Figure 7. *WT1* expression in an AML patient with CR. Representative experiment showing an AML patient with high transcripts of *WT1* during the relapse in both peripheral blood (white bars) and bone marrow (black bars). *WT1* dramatically diminished after allogeneic SCT (Tx). This low *WT1* expression is associated with the clinical CR of the patient. **Abbreviations.** d+ (number)= days after Tx, HD= healthy donor, NA= No samples were available from that specific time point.

On the other hand, the expression of *WT1* in patients that died due to the leukemia was not different before treatment to the expression in the following-up months (Figure 8) in neither peripheral blood nor bone marrow. Figure 9 shows a particular example of an AML patient with high copy numbers of *WT1* during diagnosis and relapse.

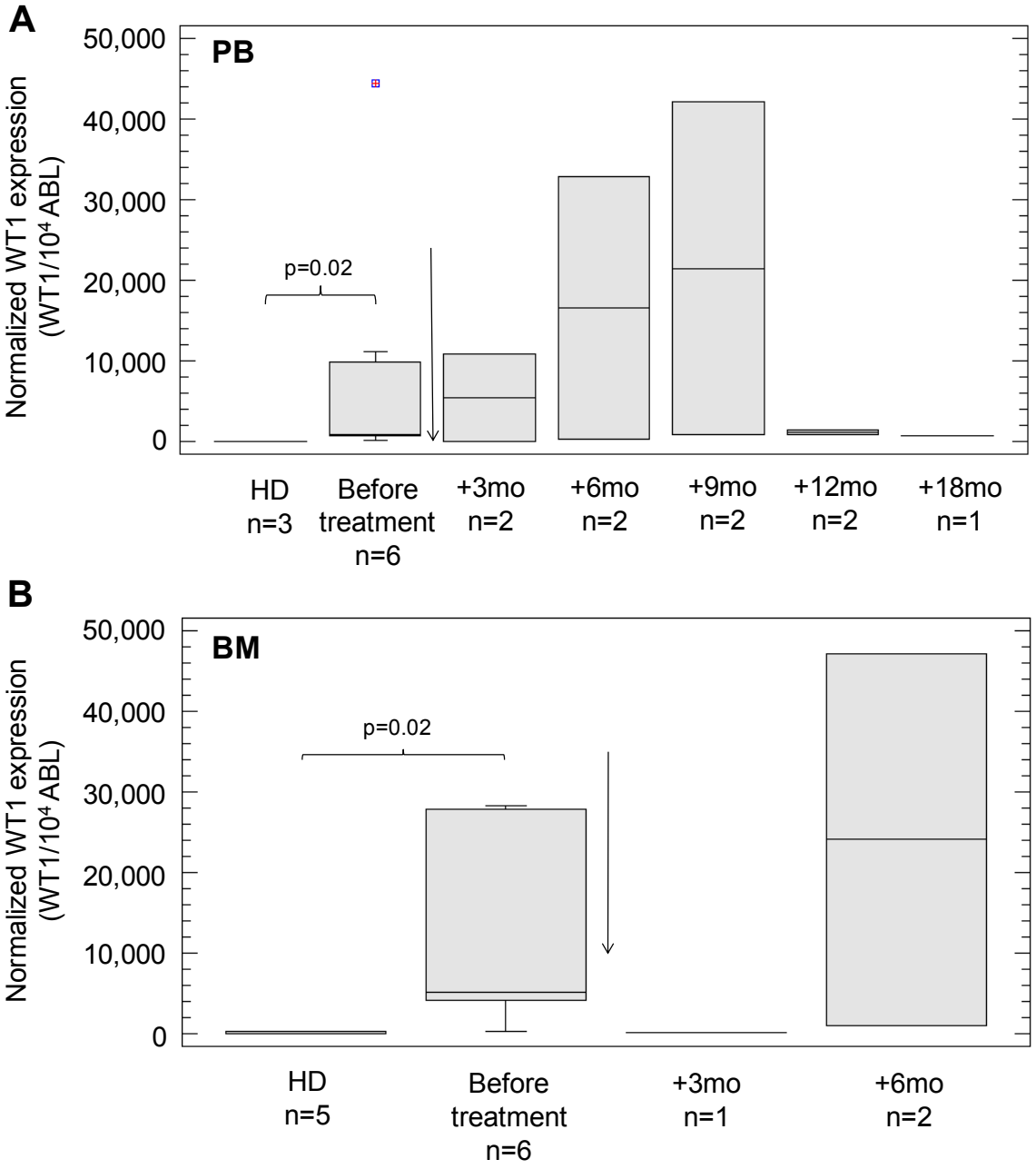

Figure 8. Expression of *WT1* transcripts in patients with an adverse clinical outcome, resulting in the death of the patients. A. There was no difference in the expression of *WT1* in the **peripheral blood** before treatment (depicted by an arrow) when compared to 3, 6, 9 and 12 months later, p=0.86, 0.86, 0.40, 0.61; respectively. Only one patient survived for 18 months. No statistical analysis can be done at this point. **B.** No significant difference was found in the expression of *WT1* in the **bone marrow** compartment before treatment compared to 6 months after (p=0.86). Only one sample was obtained 3 months after treatment. All of the patients died due to the leukemia. n refers the number of samples that were obtained at a particular following-up.

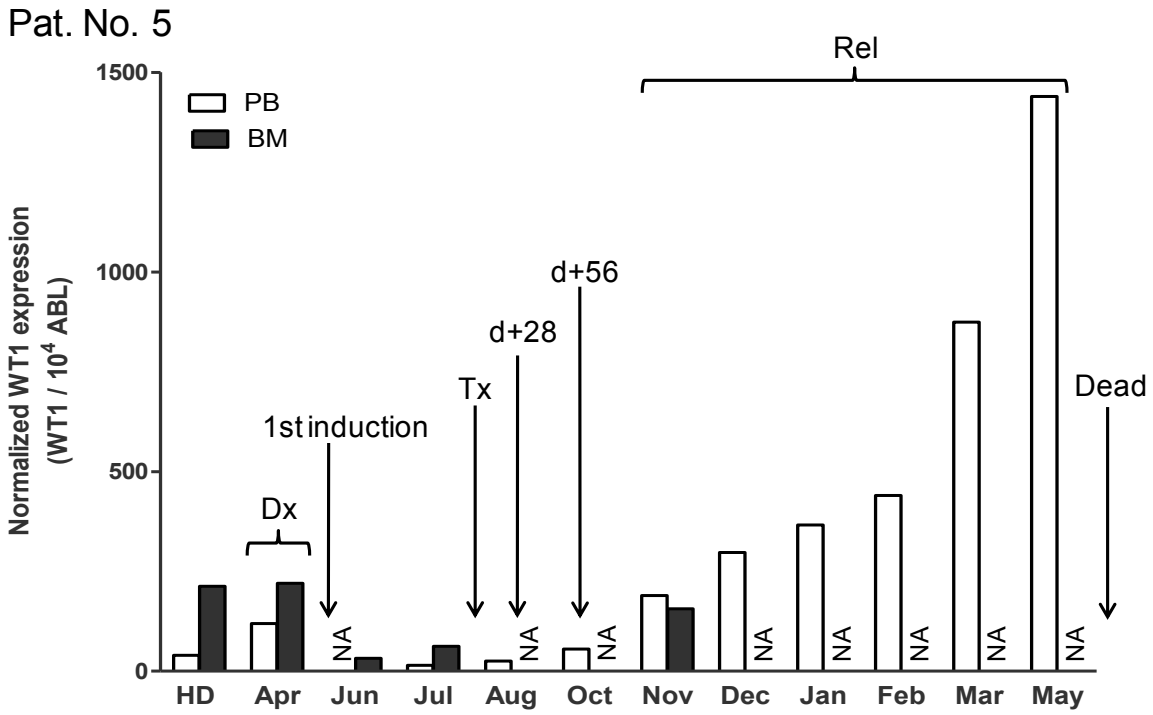

Figure 9. *WT1* expression in an AML patient with relapse of the disease. Representative experiment showing an AML patient with high transcripts of *WT1* in the peripheral blood (white bars) during the diagnosis of AML. After first induction *WT1* transcripts diminished and remained low 28 days after allogeneic SCT (Tx). However, *WT1* expression increased gradually and a clinical relapse (rel) was reported. NA= No samples were obtained at that specific time.

3.2.2 *RHAMM* transcripts

Expression of *RHAMM* in the peripheral blood of healthy donors (n=10) was very low (median: 318; range 97-730 *RHAMM* copies/10^4 *ABL* copies), whereas in patients (n=45) before treatment, the transcripts of *RHAMM* were higher (median: 810; range: 139-36160; p=0.0003) (Figure 10).

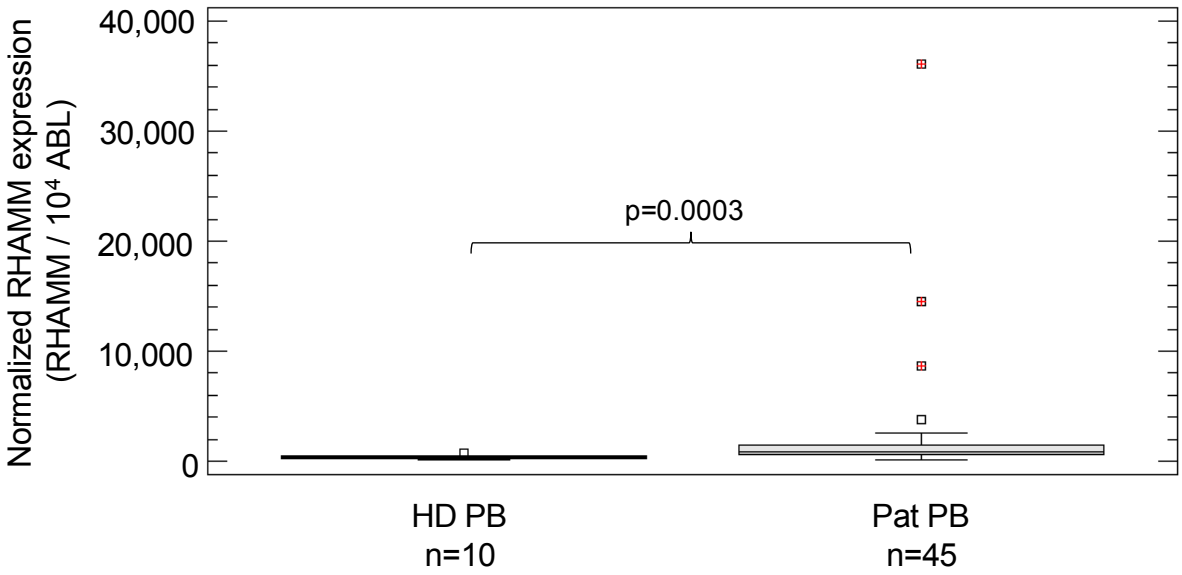

Figure 10. *RHAMM* transcripts in the peripheral blood of healthy donors and patients. *RHAMM* expression was higher in the peripheral blood of leukemia patients (Pat PB) before treatment when compared to healthy donors (HD PB, p=0.0003).

Peripheral blood was collected from 22 AML patients at the time of diagnosis. Thirteen patients were *RHAMM* positive (59%), whereas nine were negative (41%). The expression of *RHAMM* was considered positive over the highest value reached in the peripheral blood of healthy donors. *RHAMM* transcripts were significantly different (p=0.0001).

There was no significant difference in the expression of *RHAMM* in the peripheral blood of patients before treatment neither due to *FLT3-ITD* status (positive *vs.* negative, p=0.89), nor gender (p=0.66), nor different karyotypes (normal *vs.* aberrant, p=0.29; normal *vs.* complex, p=0.75; aberrant *vs.* complex, p=0.40).

Before treatment ALL patients expressed higher copy number of *RHAMM* transcripts compared to AML patients (p=0.02) in the peripheral blood. Nevertheless, transcripts of CLL were not different from those expressed by ALL (p=0.27) and AML (p=0.62).

AML, ALL and CLL patients expressed higher amount of *RHAMM* transcripts when compared to healthy donors (p=0.0009, 0.02, 0.02; respectively; Figure 11).

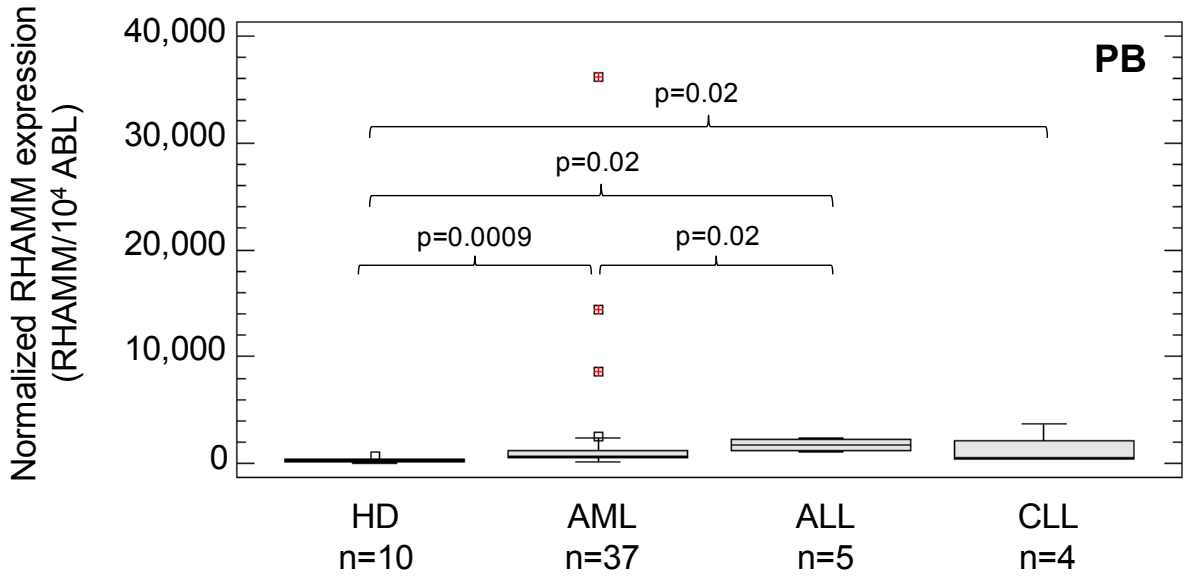

Figure 11. Expression of *RHAMM* in patients with leukemia before treatment. Patients suffering from leukemia (AML, ALL and CLL) expressed higher amount of *RHAMM* transcripts in the peripheral blood when compared to healthy donors (p=0.0009, 0.02, 0.02; respectively). *RHAMM* expression was higher in ALL than in AML (p=0.02) patients. No difference was found in the expression of *RHAMM* CLL compared to AML (p=0.62) and ALL (p=0.27).

Furthermore, we found no pattern in the expression of *RHAMM* in the peripheral blood of AML patients. Patients that were in CR at the beginning of the study had a median expression of 943 *RHAMM* copies/10^4 *ABL* copies (range: 139 - 3022) in the peripheral blood. This median of expression was similar to those who were diagnosed (737 *RHAMM* copies/10^4 *ABL*, range: 184 - 8646) and they were not statistically different from each other (p=0.79) (Figure 12).

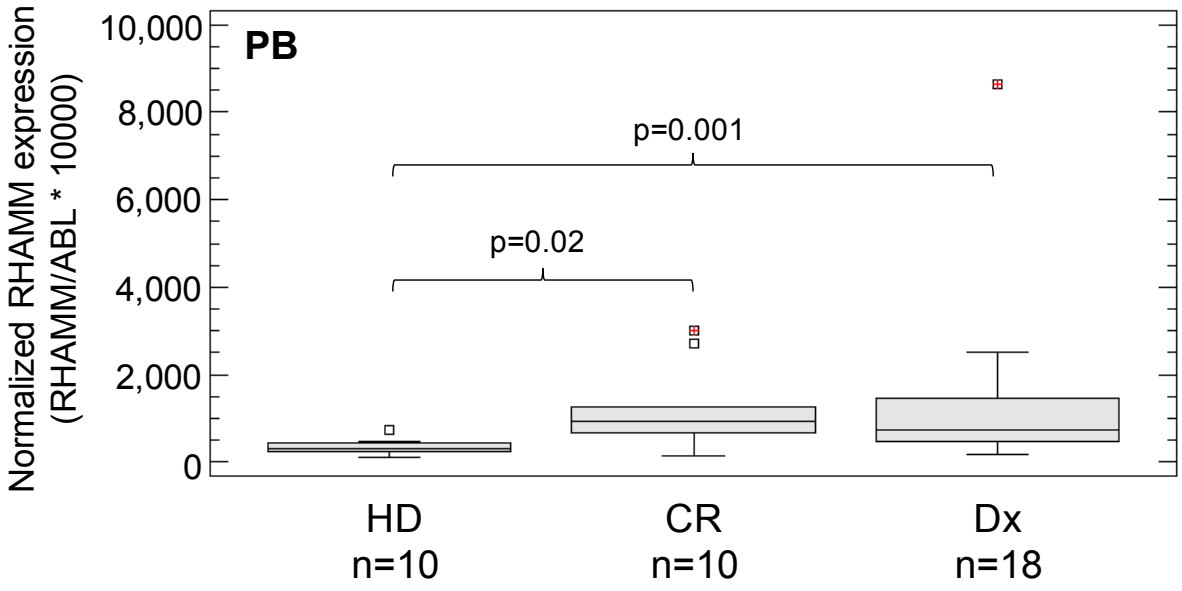

Figure 12. Normalized expression of *RHAMM* in patients in CR and at the time of diagnosis. At the time of enrollment, there was no difference in the expression of *RHAMM* in patients in CR and diagnosis in the peripheral blood (p=0.79). Nevertheless, patients in CR and diagnosis expressed higher copy numbers of *RHAMM* than healthy donors.

Figures 13 and 14 show examples of two AML patients with different expression patterns of *RHAMM*. Patient number 10 had extremely high copy numbers of *RHAMM* (higher than the cell lines OCI-AML 2 and OCI-AML 3) in the peripheral blood during PR. *RHAMM* expression was reduced during the CR, even lower than in healthy donors (Figure 13). On the other hand, *RHAMM* expression in patient number 12 was lower during the diagnosis than in the clinical CR (Figure 14).

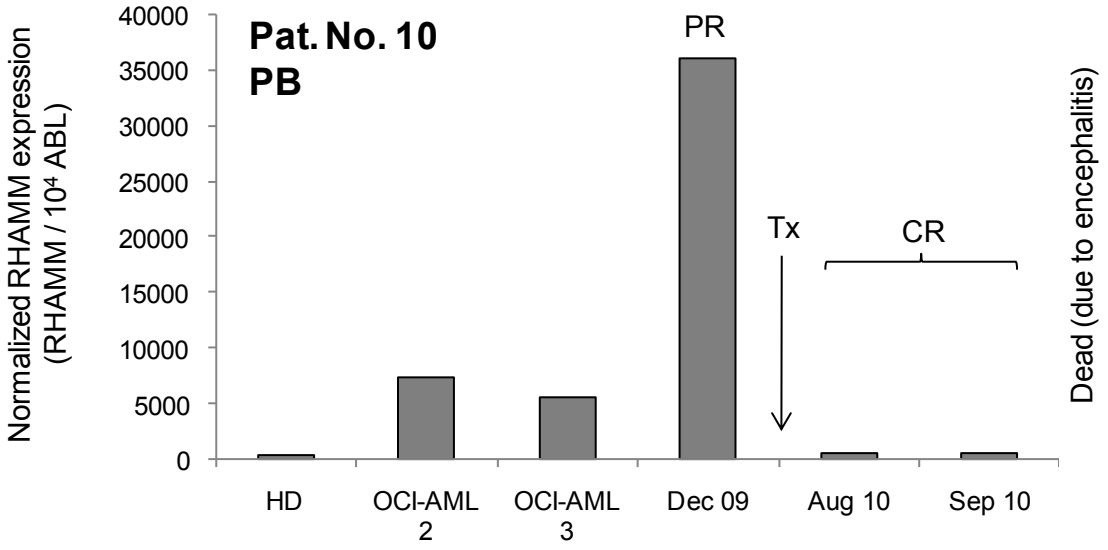

Figure 13. *RHAMM* expression in an AML patient in the course of the disease. Representative experiment showing an AML patient with high transcripts of *RHAMM* in the **peripheral blood** during PR (20% blasts in the bone marrow), higher than the cell lines OCI-AML 2 and OCI-AML 3. After allogeneic SCT (Tx) transcripts of *RHAMM* diminished drastically and the patient remained in CR until the deceased due to encephalitis of unknown origin.

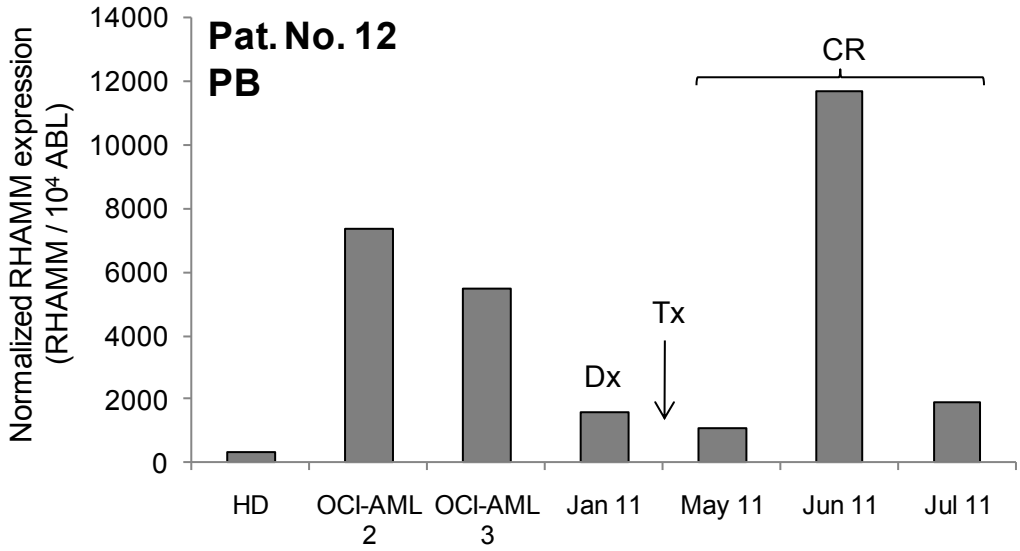

Figure 14. *RHAMM* expression in an AML patient during CR. Patient expressed higher copy numbers of *RHAMM* when compared to healthy donors (n=10). Nevertheless, *RHAMM* transcripts were higher after allogeneic SCT (Tx) when the patient was in clinical CR. Samples were obtained from the **peripheral blood** of healthy donors and from the patient.

3.2.3 Correlation of *WT1* and *RHAMM* expression

There was no correlation in the expression of *WT1* and *RHAMM* transcripts neither in peripheral blood (r^2=0.10, p=0.64) nor bone marrow (r^2=-0.18, p=0.57) of AML patients at diagnosis (Figure 15).

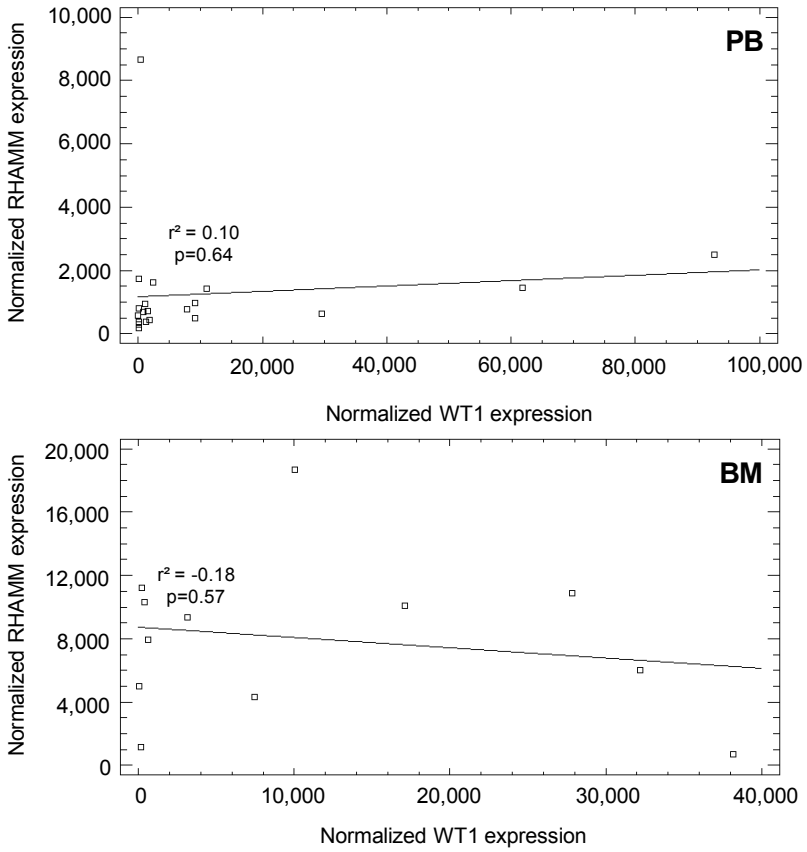

Figure 15. *RHAMM* and *WT1* expression were not correlated. Transcripts were normalized based on the expression of the house keeping gene Abelson (gene/10^4 ABL). *RHAMM* and *WT1* transcripts did neither correlate in the peripheral blood (upper panel) nor in the bone marrow of AML patients.

3.2.4 *p53* expression

p53 was highly expressed in cell lines derived from AML (OCI-AML 2 and OCI-AML 3), ALL (Nalm-6 and REH) and Burkitt's lymphoma (DG75), whereas in the CML-derived cell line (K562), *p53* was absent (Figure 16).

Expression of *p53* was higher in the peripheral blood of patients before treatment when compared to healthy donors (n=10) in the same compartment (p=0.003). Nevertheless, *p53* expression was not different in neither in different leukemias (AML *vs.* ALL, p=0.60; AML *vs.* CLL, p=0.69; ALL *vs.* CLL, p=0.76), nor in the peripheral blood of patients before and after treatment (p=0.98).

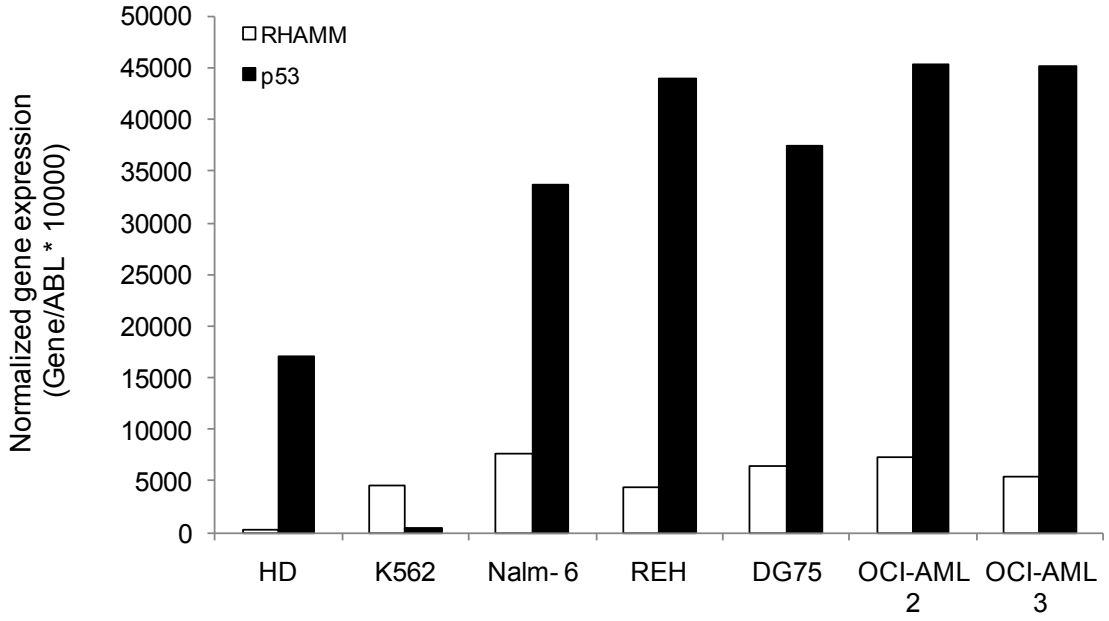

Figure 16. Expression of *RHAMM* and *p53* in different cell lines. All cell lines derived from AML, ALL and Burkitt's lymphoma had abnormal high expression of both *p53* and *RHAMM* when compared to the peripheral blood from healthy donors (n=10). *p53* was almost absent in K562.

3.2.5 Effect of *p53* on *RHAMM* and *WT1*

There was a positive correlation in the expression of *p53* and *RHAMM* transcripts in the peripheral blood (n=20, r^2=0.89, p=0.0) of AML patients during the diagnosis (Figure 17). A tendency was observed between *RHAMM* and *p53* in the bone marrow (n=12, r^2=0.50, p=0.09) of AML diagnosed patients, although it was not significant. No correlation was found in the expression of *RHAMM* and *p53* in healthy donors neither in the peripheral blood (n=10, r^2= -0.17, p=0.63) nor bone marrow (n=5, r^2=0.76, p=0.12).

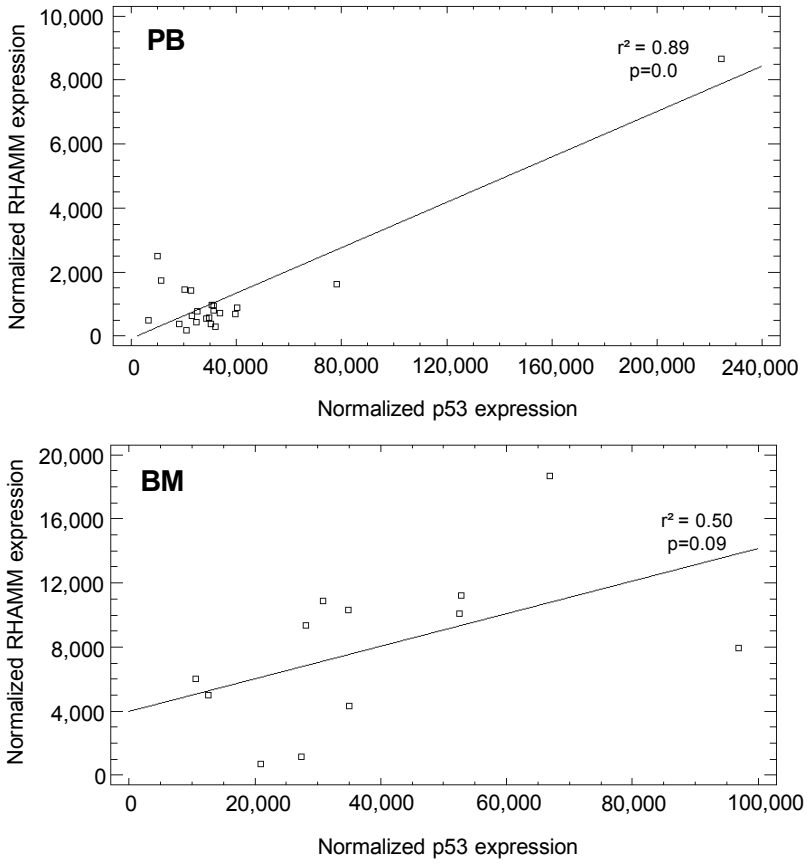

Figure 17. Positive correlation between *p53* and *RHAMM* transcripts. Transcripts were normalized with Abelson (gene/10^4 *ABL*). *RHAMM* and *p53* were positively correlated in the peripheral blood (upper panel) of 22 AML patients at diagnosis. A tendency was observed between *RHAMM* and *p53* transcripts in the bone marrow (lower panel) of 12 AML patients at diagnosis.

Moreover, there was no correlation in the expression of *p53* and *WT1*, neither in the peripheral blood (n=20, r^2= -0.21, p=0.37) nor bone marrow (n=12, r^2= -0.35, p=0.25) of AML patients during diagnosis (Figure 18).

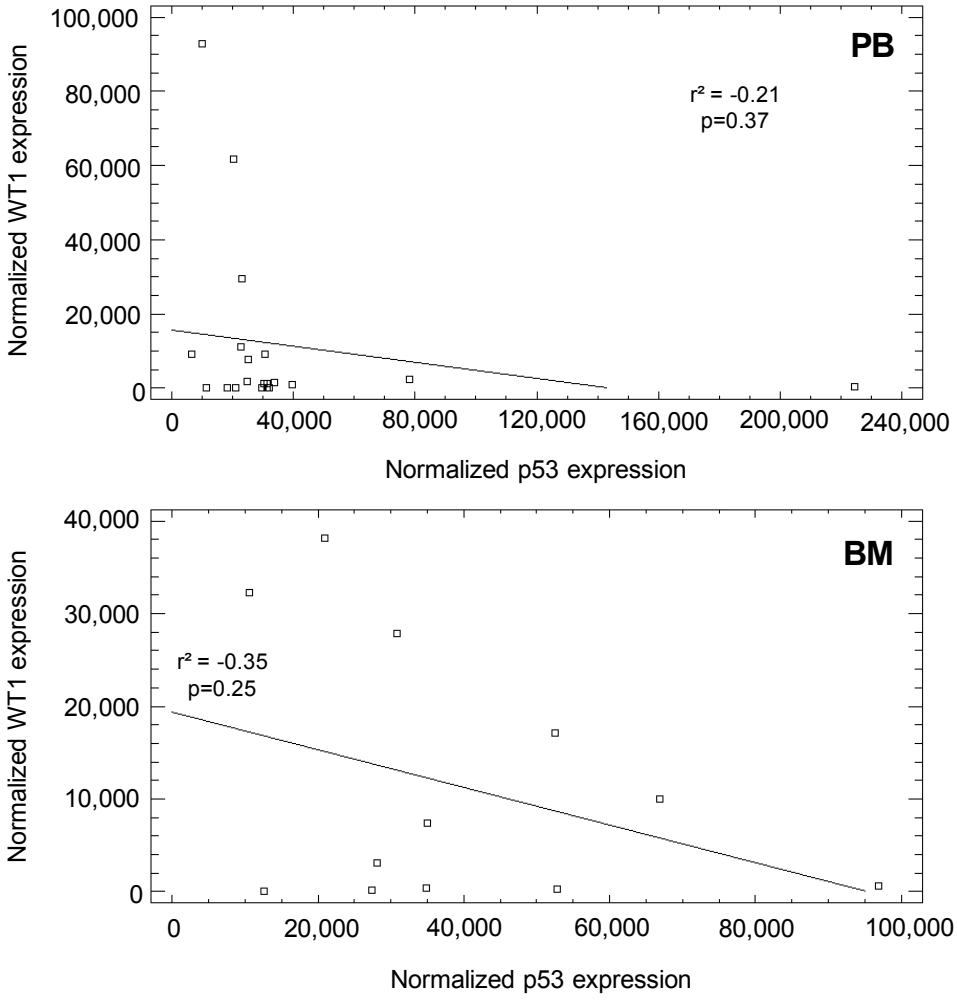

Figure 18. *p53* had no correlation to the expression of *WT1*. Transcripts were normalized with Abelson (gene/10^4 *ABL*). The expression of *WT1* and *p53* showed no correlation in the peripheral blood (upper panel) and bone marrow (lower panel) of AML patients at diagnosis.

3.3 Antigen-specific cytotoxic T lymphocytes (CTLs)

3.3.1 Antigen-specific CTLs cell responses in healthy donors

WT1-specific T cells were observed in one healthy donor by flow cytometry (HD 005, 0.17% from $CD3^+$ $CD8^+$), but no specific release of neither IFN-γ nor granzyme B was detected (Figure 19). RHAMM-specific T cells were observed in three healthy donors, HD 155 (Figure 20), 663, 005, with low frequencies (0.11%, 0.33%, 0.12%; respectively, from gate $CD3^+$ $CD8^+$). Moreover, activity of these RHAMM-specific T cells were detected in two healthy donors (HD 155 and 669) by IFN-γ (Figure 19). CTLs from healthy donor 669 were not sufficient to perform flow cytometry analysis.

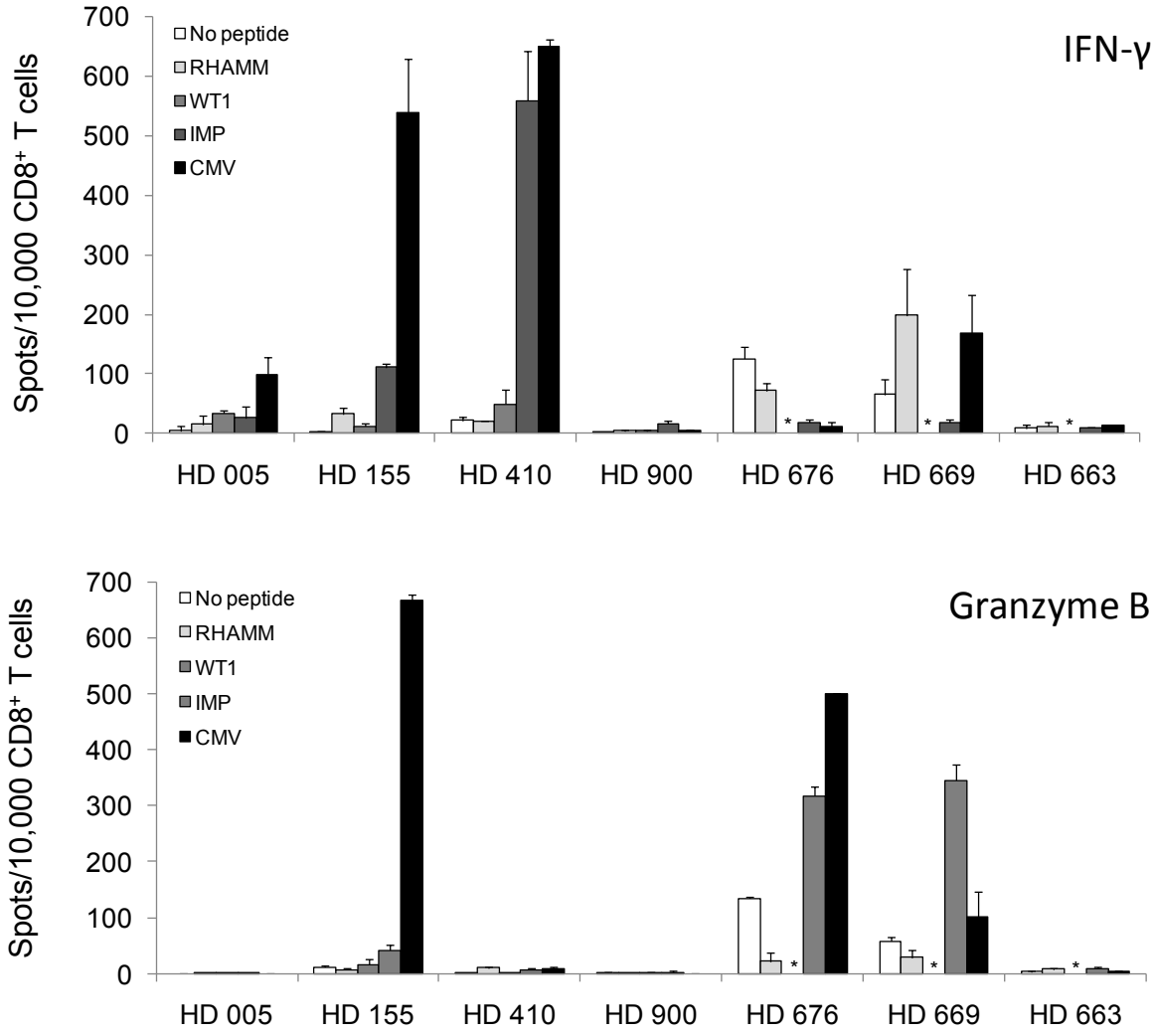

Figure 19. Antigen-specific T cells in healthy donors. Cells were stimulated in a MLPC for 7 days with different peptides and tested for their reactivity in an ELISPOT assays for IFN-γ (upper panel) and granzyme B (lower panel) release. Stimulation of the cells without any loaded-peptide was used as negative control (No peptide), whereas CMV and IMP peptides worked separately as positive controls. RHAMM-specific T cells could be detected in 2 healthy donors (HD 155 and HD 669) by IFN-γ ELISPOT. * MLPC for WT1-derived peptide was not done in healthy donors 676, 669, 663.

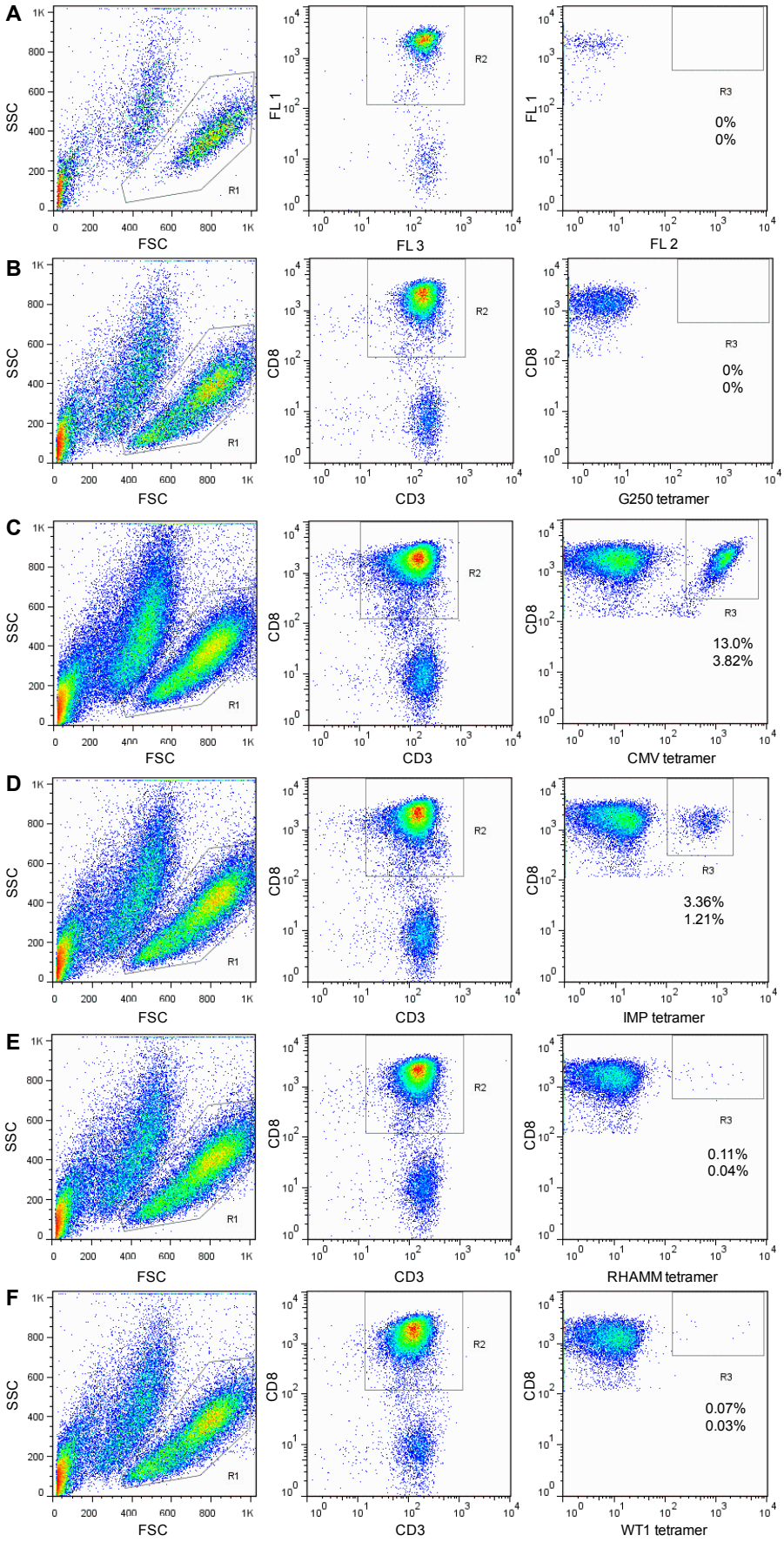
A
SSC
FSC
R1
FL 1
FL 3
R2
FL 1
FL 2
R3
0%
0%
B
SSC
FSC
R1
CD8
CD3
R2
CD8
G250 tetramer
R3
0%
0%
C
SSC
FSC
R1
CD8
CD3
R2
CD8
CMV tetramer
R3
13.0%
3.82%
D
SSC
FSC
R1
CD8
CD3
R2
CD8
IMP tetramer
R3
3.36%
1.21%
E
SSC
FSC
R1
CD8
CD3
R2
CD8
RHAMM tetramer
R3
0.11%
0.04%
F
SSC
FSC
R1
CD8
CD3
R2
CD8
WT1 tetramer
R3
0.07%
0.03%

Figure 20 (previous page). RHAMM-specific T cells detected in a healthy donor (HD 155). CTLs were stimulated in a MLPC for 7 days with different peptides. Antigen-specific T cell frequencies were determined by flow cytometry. Reported frequencies correspond to gate $CD3^+$ $CD8^+$ (upper number, R3), and from the total amount of cells (lower number, R3). **A**. Fluorescence minus one (FMO) was used as negative control to observe the intrinsic fluorescence of the cells, **B**. Non-peptide control cells were cultured in the absence of any loaded-peptide and stained with irrelevant tetramer, **C**. Positive control, CTLs were stimulated with CMVpp65 peptide, **D.** Positive control, CTLs were stimulated with IMP-derived peptide, **E**. CTLs were stimulated with RHAMM peptide, **F.** CTLs were stimulated with WT1 peptide. **C-F.** CTLs were stained with the respective tetramers.

3.3.2 Antigen-specific T cell responses in patients

3.3.2.1 WT1-specific CTLs

Activity of WT1-specific CTLs was observed in AML patients by IFN-γ and granzyme B release (Figures 21, 22 and 24). Functional WT1-specific T cells were detected by ELISPOT in $WT1^+$ AML patients during the relapse (Figure 21) and at the time of diagnosis (Figures 22). Unfortunately, the amount of cells was not sufficient to perform flow cytometry analysis at these points. Nevertheless, high frequencies of WT1-specific T cells were observed by flow cytometry (Figures 23, S2, S4 and S5) in the peripheral blood of the patient after chemotherapy (Pat. No. 5). IFN-γ and granzyme B ELISPOT assays reveal lack of functionality of these CTLs (Figures 21 and 22). This population was lost over the time (Figures 23, S2, S4 and S5). This may explain partially the relapse of the patient.

Interesting was the fact that functional WT1-specific T cells were observed in a $WT1^-$ AML patient in both peripheral blood and bone marrow (Figure 24) during the CR. These CTLs reside in the bone marrow of patients during CR (Figures 21 and 24) independently of chemotherapy (Figures 21).

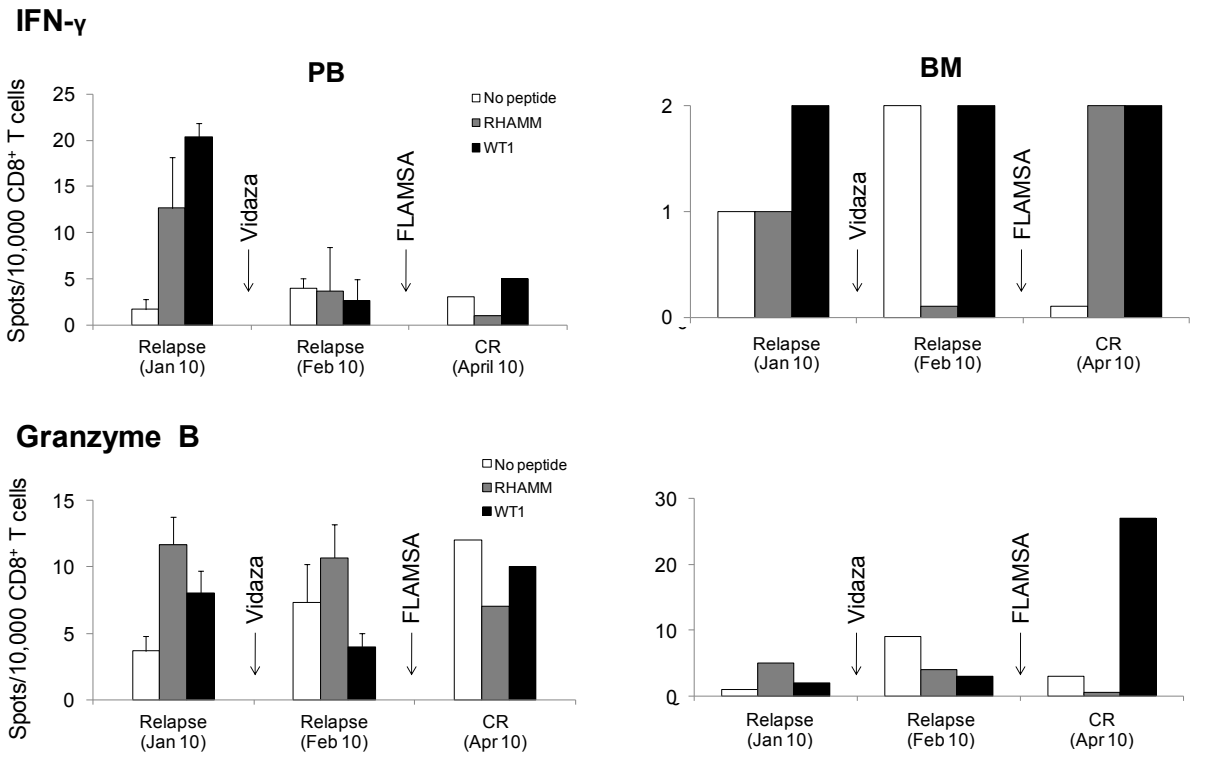

Figure 21. WT1-specific T cells in a WT1$^+$ patient (Pat. No. 4) during relapse of AML. Both IFN-γ (left, upper panel) and granzyme B (left, lower panel) release from RHAMM- and WT1-specific T cells was detected by ELISPOT in the peripheral blood of a patient during relapse (January, 2010). This population was lost following Vidaza treatment (February, 2010). Activity of WT1-specific T cells was observed by granzyme B in the bone marrow (right, lower panel) of the patient at the time of CR. Cells were not enough to perform flow cytometry analysis.

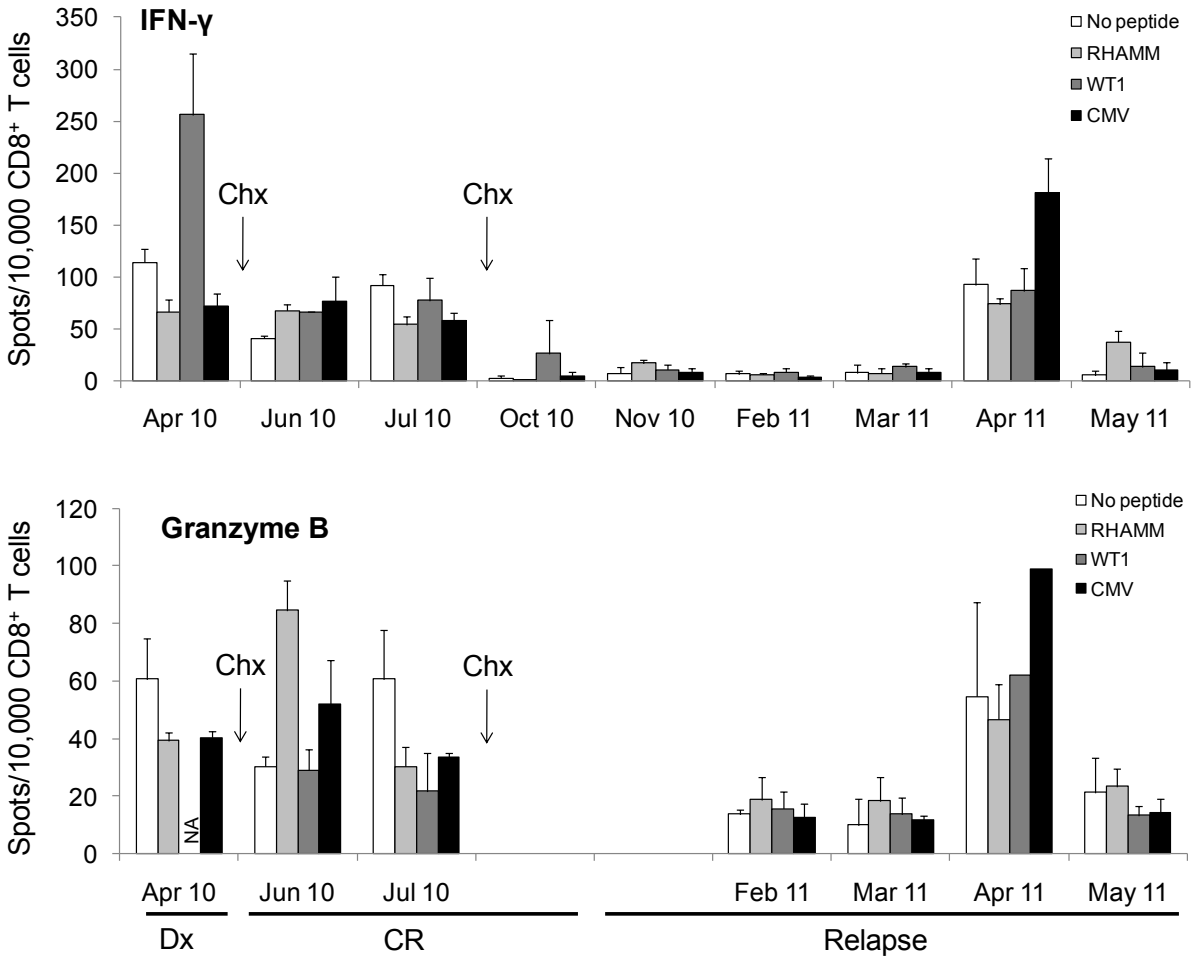

Figure 22. WT1-specific T cells in a WT1$^+$ patient (Pat. No. 5) during diagnosis of AML. CTLs were obtained from the peripheral blood of the patient. Activity of WT1-specific T cells was observed by IFN-γ release (upper panel) during diagnosis of AML. Such population was lost after the induction chemotherapy (Chx: Idarubicin, Ara-C). RHAMM-specific T cells detected by granzyme B during the diagnosis and lost over the time.

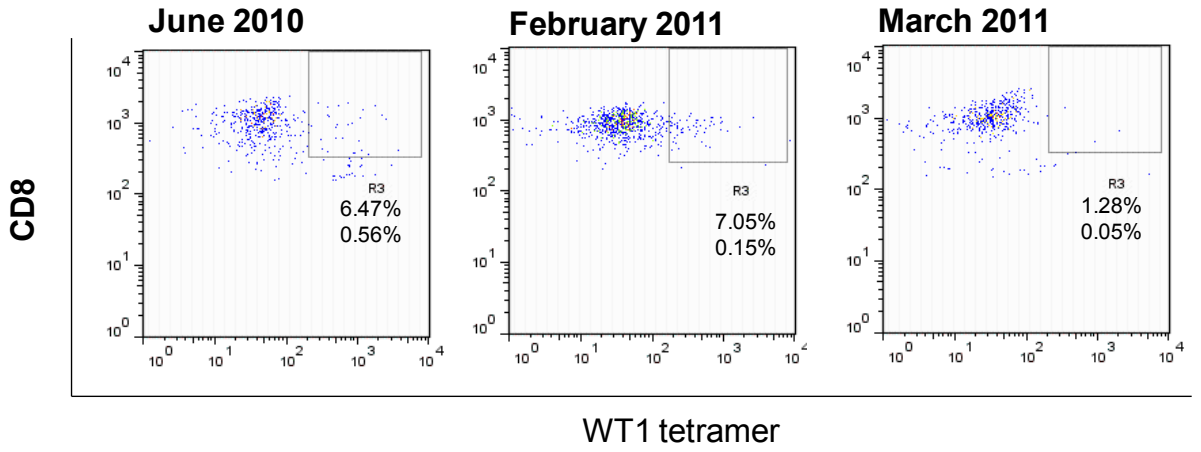

Figure 23. WT1-specific T cells in a WT1$^+$ patient during the course of the disease (Pat. No. 5). Samples were collected from the peripheral blood of the patient during CR (June 2010) and at relapse (February and March 2011) of the patient. The frequency of WT1-specific T cells is reduced over the time in the peripheral blood. Reported frequencies correspond to gate CD3$^+$ CD8$^+$ (upper number), and from the total amount of cells. CTLs were stimulated with WT1 peptide and stained with respective tetramer (See Supplement Figures S2, S4, S5).

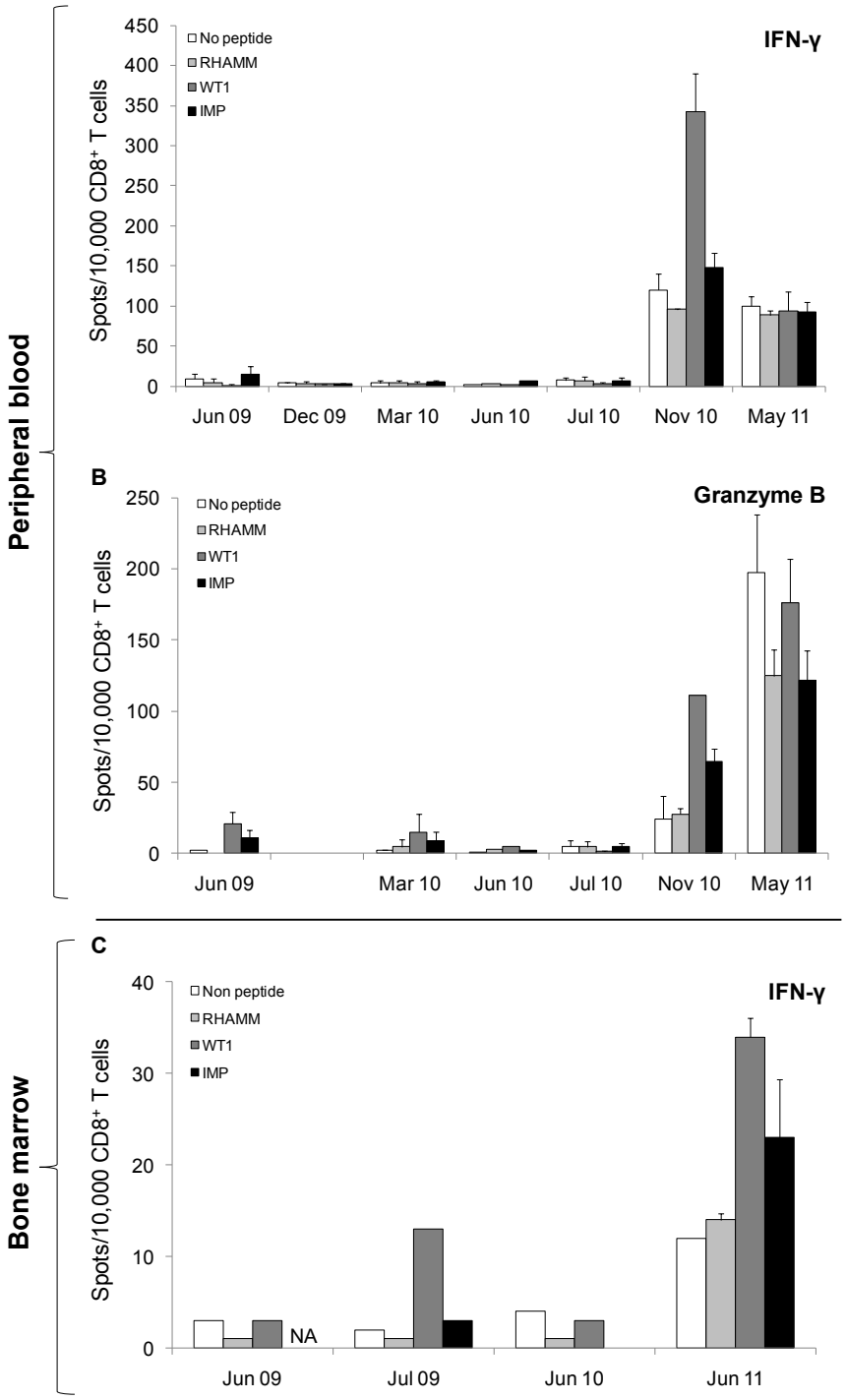

Figure 24. WT1-specific T cells in a WT1$^-$ AML patient (Pat. No. 2) in CR. Samples were obtained from the peripheral blood (**A** and **B**) and from the bone marrow (**C**) of a chemotherapy-free patient. Activity of WT1-specific T cells could be detected by both IFN-γ (**A**) and granzyme B (**B**) ELISPOT assays (November 2010) in the peripheral blood. The activity is vanished by May 2011. Moreover, WT1-specific T cells are found in the bone marrow (**C**) in July 2009 and two years later in June 2011. Cells were not sufficient to perform flow cytometry analysis in parallel.

3.3.2.2 RHAMM-specific CTLs

Frequencies of RHAMM-specific T cells were observed by flow cytometry in five AML patients (Pat. No. 5, 6, 43, 51, 57; Figures 25, S2-S5, 27 and 28). However, these population was non functional as demonstrated by the absence of granzyme B and IFN-γ release in the ELISPOT assays in two patients (Figures 22 and 26). Unfortunately, we could not contrast this finding by flow cytometry on those RHAMM-specific T cell that were demonstrated to be functional by ELISPOT due to the lack of cells (*i.e.* Pat. No. 4; Figure 21). Moreover, RHAMM-specific T cells were diminished after chemotherapy (Figures 21, 22, 25, S2-S5, 27 and 28) and vanished over the time (Figures 25, S2-S5).

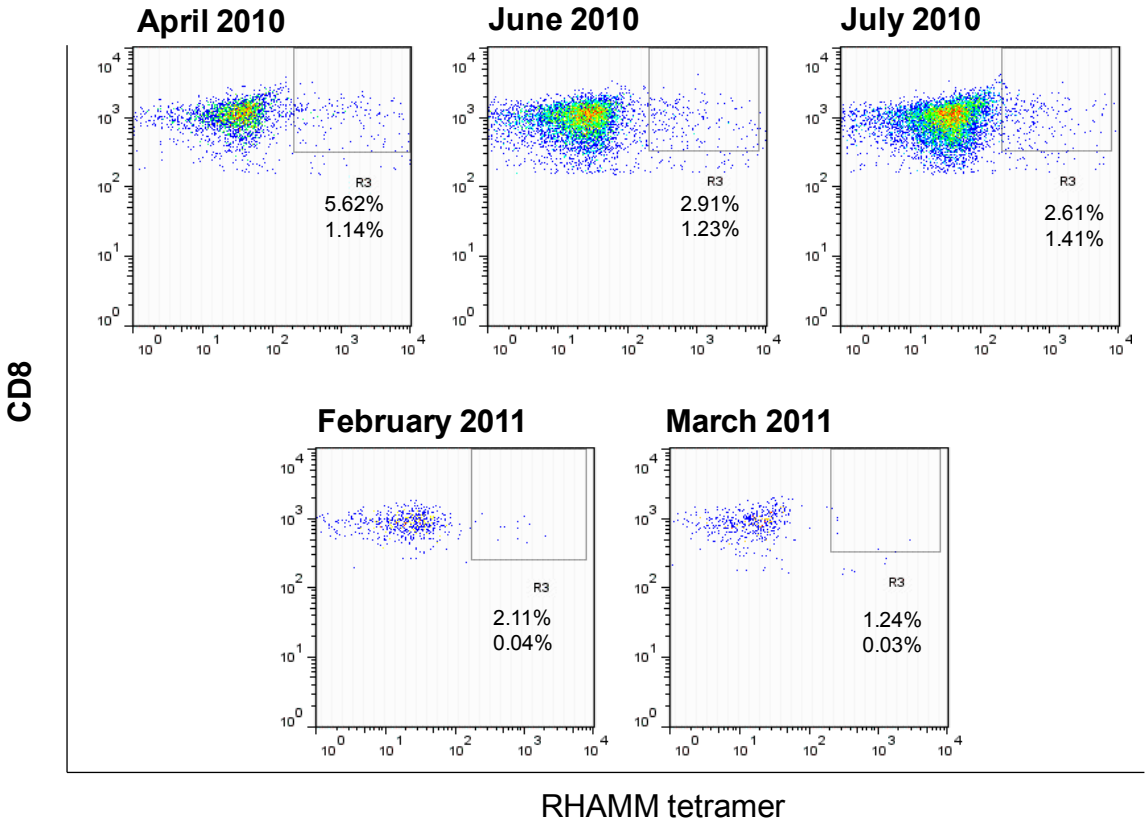

Figure 25. RHAMM-specific T cells in a RHAMM$^+$ patient during the course of the disease (Pat. No. 5). Samples were collected from the peripheral blood of the patient during the time of diagnosis (April 2010), CR (June and July 2010) and at relapse (February and March 2011) of the patient. RHAMM-specific T cells were vanished over the time. Reported frequencies correspond to gate CD3$^+$ CD8$^+$ (upper number, R3), and from the total amount of cells. CTLs were stimulated with RHAMM peptide and stained with respective tetramer (See Supplement Figures S1-S5).

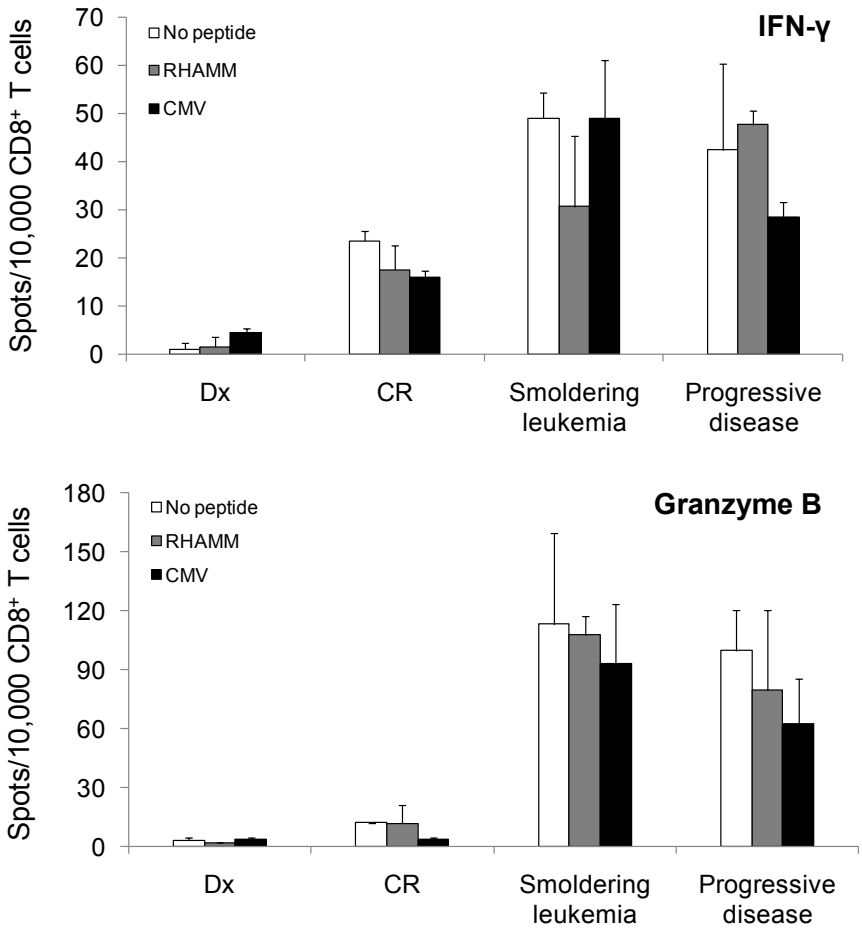

Figure 26. RHAMM-specific T cells are not functional (Pat. No. 51). RHAMM-specific T cells were observed by flow cytometry in the smoldering leukemia (Figure 27) and progressive disease (Figure 28), but they are non functional as demonstrated by ELISPOT assays.

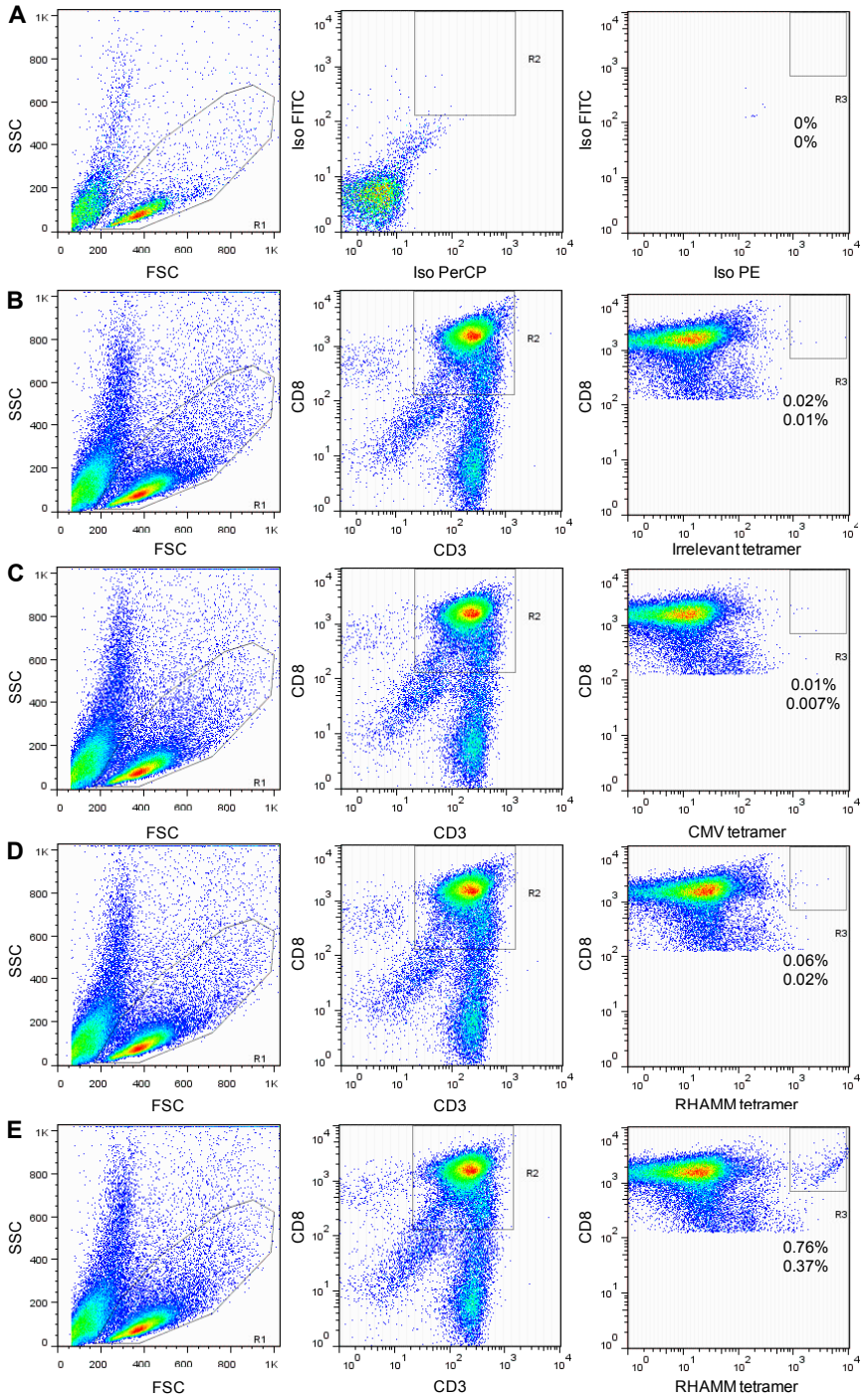

Figure 27. RHAMM-specific T cells in a RHAMM$^+$ patient (Pat. No. 51) during smoldering leukemia. Samples were obtained from the peripheral blood of the patient. Reported frequencies correspond to gate CD3$^+$ CD8$^+$ (upper number, R3), and from the total amount of cells (lower number, R3). **A.** Isotype, **B**. Non-peptide control, stained with irrelevant tetramer, **C.** Positive control, CTLs were stimulated with CMVpp65 peptide and stained with CMV tetramer **D.** Non-peptide control, stained with RHAMM tetramer, **E.** CTLs were stimulated with RHAMM peptide and stained with RHAMM tetramer.

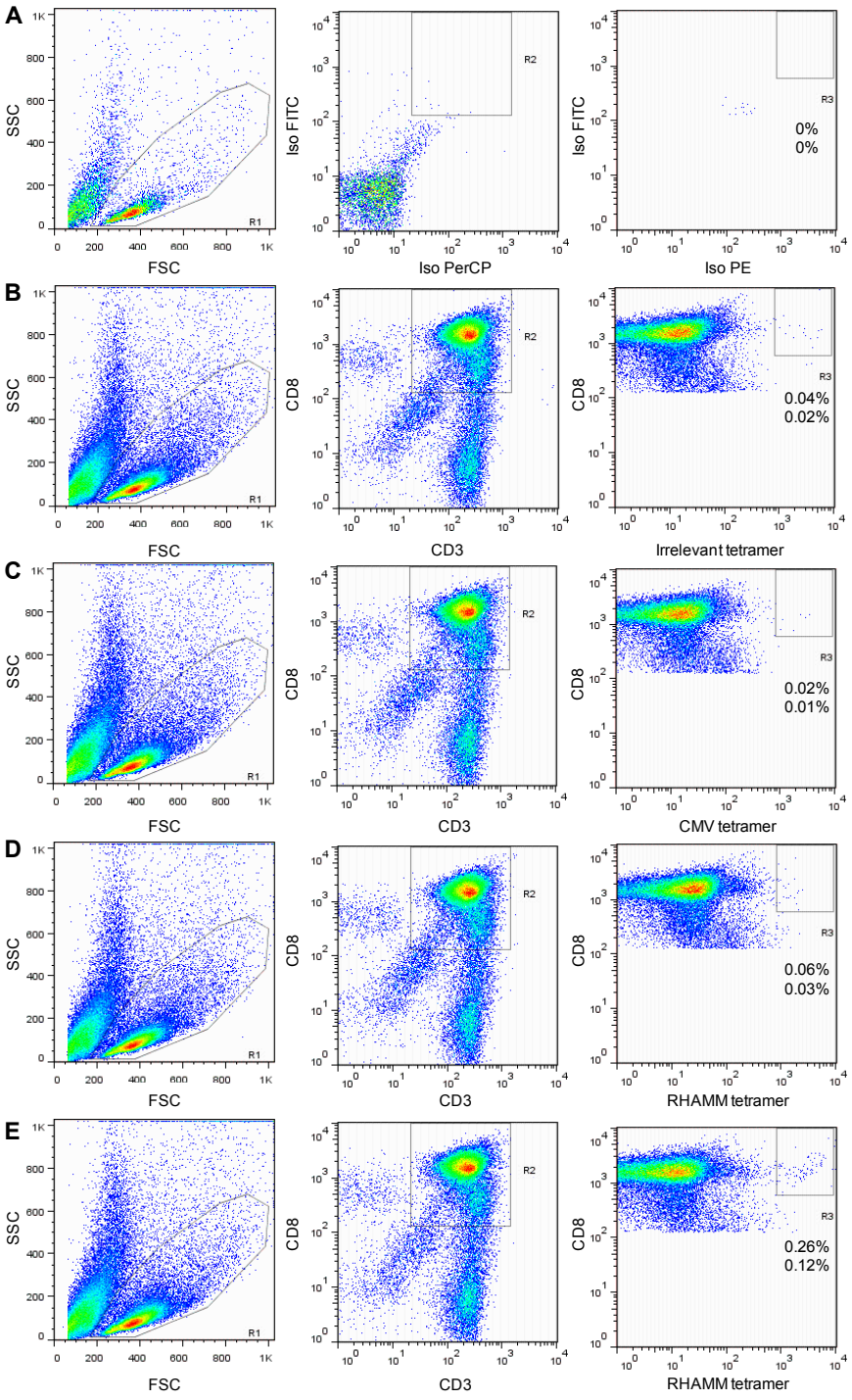

Figure 28. RHAMM-specific T cells in a RHAMM$^+$ patient (Pat. No. 51) during progressive disease. Samples were obtained from the peripheral blood of the patient after Litalir treatment. Reported frequencies correspond to gate CD3$^+$ CD8$^+$ (upper number, R3), and from the total amount of cells (lower number, R3). **A**. Isotype, **B**. Non-peptide control, stained with irrelevant tetramer, **C**. Positive control, CTLs were stimulated with CMVpp65 peptide and stained with CMV tetramer, **D**. Non-peptide control, stained with RHAMM tetramer, **E**. CTLs were stimulated with RHAMM peptide and stained with RHAMM tetramer.

3.4 Cytokine expression

Although we found no statistical difference in the cytokine milieu before and after treatment (n=8 patients), we could observed some individual differences in the patients with a clinical good outcome (Figure 29) as well as in patients that deceased (Figure 30) due to the disease. Two examples of each case are depicted in Figures 29 and 30.

Patients with a clinical good outcome had different expression of cytokines compared to those patients who died due to the disease. Patients that maintained CR (*i.e.* Pat. No. 6 and 22) showed a high expression of IL-2, IL-17, GM-CSF, TNF-α and MIP-1β not only when leukemic blasts were presented (PR and relapse), but also during CR, although the levels diminished. On the other hand, TNF-α was not expressed at all in those patients who died due to the disease. CXCL10 was expressed in all of the patients during relapse. However, only in patients with a clinical good outcome, CXCL10 was presented during the CR. IL-8 was highly expressed in three patients during relapse (Pat. No. 4, 6 and 22), but only in those with good clinical outcome, IL-8 remained during CR at lower levels. Moreover, CD40 ligand was expressed by patients with a clinical good outcome, with higher expression during CR. During the CR IL-1ra was lower in patients with bad outcome (Pat. No. 4 and 5), compared to one patient with good outcome (Pat. No. 22). sTREM diminished in CR in a patient with clinical good outcome (Pat. No. 6), whereas in the one that die due to leukemia (Pat. No. 5) it augmented.

C5a was highly expressed in 4 patients (4/8) when blasts were found. It diminished during CR (Figures 29A and 30A). Unexpectedly, IFN-γ was low or not expressed neither during relapse nor during CR in none of the patients (n=8). IL-1β, IL-4, IL-5, IL-10, IL-32α and CCL2 were highly expressed only by one patient (1/8, Pat. No. 6) during PR and their expression diminished in the CR of the patient. CXCL12 and IL-12p70 were also expressed during PR and their expression diminished in the CR.

IL-6 and IL12p70 was expressed by two patients (Pat. No. 4 and 6, and Pat. No. 6 and 22; respectively) during the presence of blast and diminished in the CR.

Of special interest was the observation of IL-23, IL-17E, CCL1 and IL-13 expressed only by one patient (1/8, Pat. No. 5) during clinical CR. This patient died due to the disease (Figure 30B).

MIP-1α and CXCL 11 were not expressed at any point. sICAM-1, MIF, serpin E1 and RANTES were highly expressed during relapse (and PR) and in CR in all of the patients (n=8).

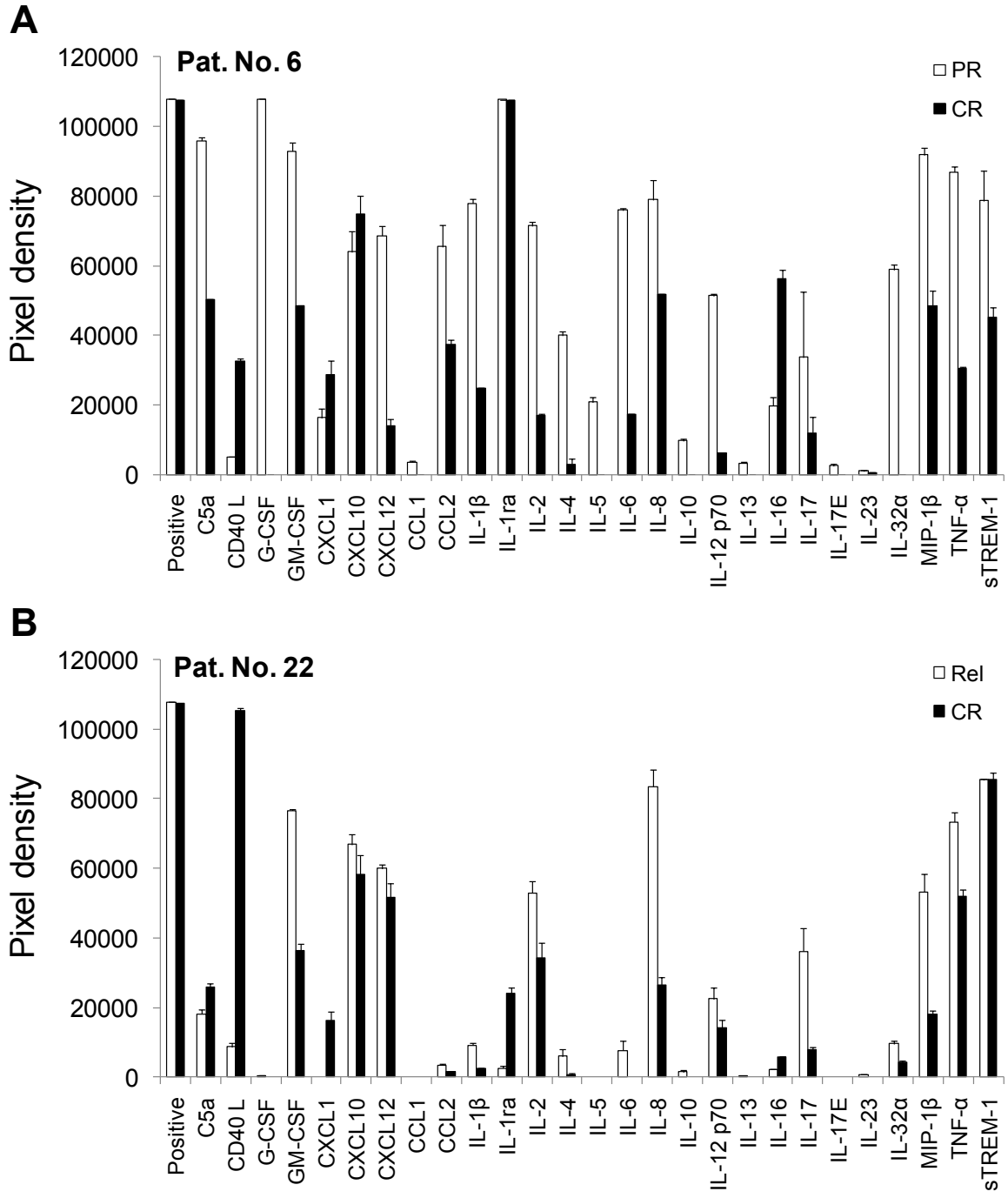

Figure 29. Cytokine expression in two AML patients with a clinical good outcome. A. C5a, G-CSF, GM-CSF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-17, IL-32α, CCL2, MIP-1β, CXCL12, TNF-α and sTREM-1 were reduced when the patient reached CR, whereas CD40 ligand, CXCL1 and IL-16 increased. Samples were obtained before (PR) and after (CR) a 2nd allogeneic STC, **B.** Higher expression of GM-CSF, IL-2, IL-8, IL-17, MIP-1β and TNF-α was detected during the relapse, whereas the expression of CD40 ligand, CXCL1 and IL-1ra was lower. Samples were obtained before (relapse) and after (CR) allogeneic STC. Differences were considered when pixel density was bigger than 10,000.

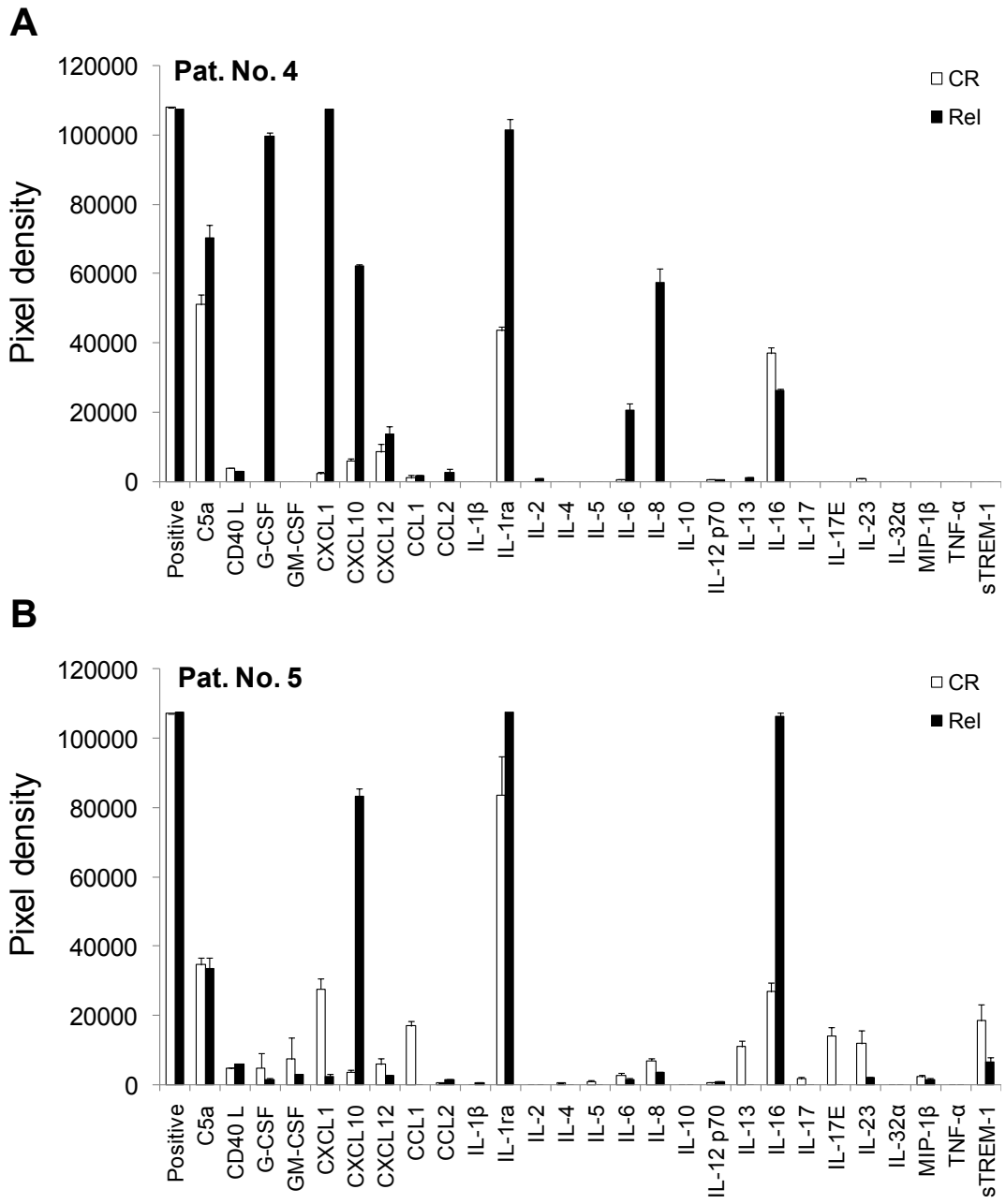

Figure 30. Cytokine expression of two AML patients with an unfavorable clinical outcome (deceased due to leukemia). A. The patient had allogeneic SCT after 1st relapse. CR was reached and a second relapse was observed. Samples correspond to the second relapse with the highest copy numbers of WT1 transcripts. The expression of C5a, G-CSF, CXCL1, IL-1ra, IL-6, IL-8 and CXCL10 was lower during the CR, while IL-16 was higher, **B**. Patient received chemotherapy and reached CR, followed by allogeneic SCT. The patient relapsed 3 months after allogeneic SCT. CXCL1, CCL1, IL-13, IL-17E, IL-23 and sTREM-1 levels decrease during the relapse, whereas IL-1ra, IL-16 and CXCL10 increased. Differences were considered when pixel density was bigger than 10,000

4 DISCUSSION

In this work we evaluated the expression of LAAs in 69 patients suffering from leukemia. We investigated the expression of *WT1* and *RHAMM* at a RNA level using RQ-PCR in patients before and in the following-up after treatment. Additionally, we tracked specific CTLs for these antigens. These molecular and immunological parameters were correlated with the clinical status of the patients.

4.1 RNA expression of LAAs

The expression of *WT1* in the peripheral blood and bone marrow of healthy donors was comparable to previous reports by other groups (Cilloni *et al.* 2008, Cilloni *et al.* 2009, Nowakowska-Kopera *et al.* 2009). A key result that confirms the competence of our technique. Moreover, no significant difference was found in the expression of *WT1*, neither in the peripheral blood nor in the bone marrow, respect to the *FLT3-ITD*, karyotype and sex. Our findings are in concordance with previous reports from Cilloni *et al.* (2008) and Nowakowska-Kopera *et al.* (2009).

Furthermore, patients in CR had an expression of *WT1* as low as healthy donors. On the other hand, reoccurrence of the *WT1* signal preceded an imminent relapse. According to our findings and others (Cilloni *et al.* 2008, Cilloni *et al.* 2009, Nowakowska-Kopera *et al.* 2009), *WT1* represents a useful marker to monitor MRD. Currently, *WT1* expression analysis is not part of routine laboratory tests in clinical practice. Since a big ratio of the patients suffering from leukemia are $WT1^+$ (88% of the *de novo* AML patients were $WT1^+$ in our cohort), we suggest that *WT1* should be measured periodically in patients in order to detect any evidence of MRD. Consistently proven by others (Cilloni *et al.* 2008, Cilloni *et al.* 2009, Nowakowska-Kopera *et al.* 2009), as well as by this report, WT1 is a trustful MRD marker that can predict a clinical relapse from three weeks to three months.

Furthermore, Bergmann *et al* (1997a) reported that expression of WT1 is more likely to be expressed in AML blast that expressed other LAAs. Here, we found no correlation in the expression of *WT1* and *RHAMM* at RNA level.

On the other hand, little is known about the prognostic role of RHAMM and its interaction partners in leukemia. Tzankov *et al.* (2011) analyzed RHAMM expression at the protein level on bone marrow biopsies of a large cohort of AML patients. They found that 28% of the patients were RHAMM positive and that RHAMM could be a good prognostic factor at the protein level. However, no systematic study has been done to investigate the role of RHAMM as a prognostic factor at the RNA level. In the present study, we established a routine procedure to quantify absolute copy numbers of *RHAMM* using RQ-PCR.

The expression of *RHAMM* was not significantly different with respect to the *FLT3-ITD* status, karyotype and sex in the peripheral blood patients. In this study, 59% of the *de novo* AML patients were RHAMM^{+}. This finding is consistent with work by Greiner *et al.* (2004). They reported 70% (35/50) of the AML patients were positive for *RHAMM* as determined by conventional RT-PCR. However, some of the patients that were RHAMM negative during the diagnosis became positive during the reported clinical CR, as confirmed in this study by the absence of *WT1* transcripts. Furthermore, another cohort of patients that were RHAMM positive during the diagnosis became negative during the reported clinical CR. Based on the data from this report we cannot support the idea of *RHAMM* as a MRD marker since some of the patients from our cohort expressed *RHAMM* indistinctly of the clinical status. However, *RHAMM* transcripts may be a useful tool to determine the presence of leukemia blast during the diagnosis.

Sohr and Engeland (2008) reported that p53 downregulates the expression of mRNA *RHAMM* and also at the protein level. They conducted studies on human colorectal carcinoma cell lines. Our results showed a positive correlation of *p53* and *RHAMM* in the peripheral blood of patients. A trend was observed in the bone marrow compartment, but it was not significant (p=0.09). Nevertheless, this may not suggest RHAMM as a target of p53 in leukemias, but a lack of function of p53. More detailed studies involving the knock-down or the overexpression of these genes in leukemia cell lines are necessary to solve this problem.

In the past the absence of p53 mutations in the majority of hematological malignancies has been reported. These reports may be targeting critical target genes

inactivated in tumor cells. Here, we found high expression of *p53* in cell lines derived from AML and ALL blasts, as well as in primary leukemia blasts from patients. Our results are comparable to those found by Prokocimer *et al.* (1986) in cell lines, which can support the proficiency of our method. This finding suggests that even thought p53 is overexpressed in leukemias, it may be either not functional or the p53 cascade has a defect. An idea that has been proposed by Boyapati *et al.* (2004) and recently re-took by Prokocimer and Peller (2011).

4.2 LAAs-specific CTLs

We hypothesize that the presence of LAAs-specific T cells may be partially involved in the maintenance of the CR of patients.

Functional RHAMM- and WT1-specific CTLs were detected in AML patients by ELISPOT. This population vanished in the peripheral blood of patients after they received chemotherapy and over the time in a patient that relapse. Potential down-regulatory effects of chemotherapy on T cells have been reported previously (Seggewiss *et al.* 2005, Chen *et al.* 2007, Chen *et al.* 2008).

Nevertheless, the observation of functional WT1-specific CTLs in the bone marrow of patients during CR suggested that *i)* the hematopoietic microenvironment protects these antigen-specific T cells and *ii)* the existence of central memory T cells for the WT1 antigen. Not supportive experiments to proof these hypotheses were made in

this report due to the restriction in the amount of cells. Detailed studies to determine the phenotype of these CTLs, such as CCR7 and CD45-RA, need to be performed.

WT1-specific CTLs were observed in patients that were not WT1 positive (*i.e.* patient No. 2). This finding suggests that CTLs specific for other antigens may recognize partially or the full WT1-derived peptide (RMF PNA PYL), an event known as cross-reactivity. Cross-reactivity has been reported for diverse virus epitopes (Cao *et al.* 1997, Mani *et al.* 2011). Moreover, CTLs can cross-react with antigens originated from virus and self peptides (Kuzushima *et al.* 1995, Misko *et al.* 1999, Mason *et al.* 2005), as well as with bacterial and self peptides (Misko *et al.* 1999). This promiscuous specificity has been reported also for mycobacterial proteins and self peptides (Zügel *et al.* 1995). This cross-reactivity may explain the presence of WT1-specific T cells in healthy donors found by Wang *et al.* (2010). WT1 is expressed in the very early stage of $CD34^+$ stem cell cultures, but decreases rapidly when cells expressed CD33 surface antigen (Maurer *et al.* 1997).

4.3 Cytokine milieu

The cytokine milieu surrounding T cells may be crucial for their expansion and activation. We observed a prevalence of inflammatory cytokines in the serum of patients when blasts were presented. It is well known that chronic inflammation promotes tumor growth (Jiang *et al.* 2010). For example C5a, which triggers inflammation, diminished during the CR. This may be partially explained by *in vitro* studies conducted by Orr *et al.* (1979). They showed that a derivative peptide of C5a

attracted tumor cells by chemotaxis. Although, this may be look careful since C5a is also involved in complement activation in normal conditions. Nevertheless, this inflammatory and T cell stimulatory milieu might contribute to the favorable outcome of patients.

G-CSF and GM-CSF were highly expressed by patients during PR and relapse. These cytokines are produced and released by leukemia blasts, thus stimulating their growth and proliferation. CXCL10, also known as γ-IFN inducible protein 10 (IP 10) was expressed by all of the patients during relapse or PR. But only in patients with a clinical good outcome it was continuously expressed in the clinical CR. Since CXCL10 is a chemoattractant of T helper 1 (Th1) lymphocytes it may indicate supportive assistance to CTLs. Th1 cells also produce IL-2, which was highly expressed in patients with a clinical good at the time of CR.

IL-2, TNF-α and MIP-1β were highly expressed in patients that had a clinical good outcome not only during the relapse and PR, but also during CR. IL-2 is produced by T cells, both $CD4^+$ and $CD8^+$, in response to antigenic stimulation and it is required for T cell proliferation. Currently, IL-2 is used as supportive treatment for patients with cancer. In our R3 vaccination trial (Schmitt *et al.* 2008), we detected elevated levels of IL-2 in the serum of patients with a positive clinical response to vaccination. TNF-α is a pro-inflammatory cytokine secreted by CTLs, and other cells, and it is cytotoxic to tumor cells. MIP-1β is a potent chemoattractant for dendritic cells and B cells, APCs that are involved in the presentation of antigens. Patients that die due to the disease did not express any detectable IL-2, TNF-α nor MIP-1β.

4.4 Concluding remarks

We concluded that *WT1* is a suitable marker for MRD after allogeneic SCT and that a WT1-specific T cell response might contribute to the maintenance of CR. Moreover, we cannot support *RHAMM* as a MRD marker. Furthermore, specific T cell responses against LAAs, such as RHAMM and WT1 can be raised and these specific CTLs may be raised from cross-reactivity. In addition, an inflammatory and T cell stimulatory cytokine milieu might contribute to the favorable outcome of patients. Relapses predicted by RQ-PCR for WT1 could be prevented by immunotherapy approaches such as antigen-specific DLIs and peptide vaccination.

5 LITERATURE (in alphabetical order)

1. Abetamann V, Kern HF, Elsässer HP. Differential expression of the hyaluronan receptors CD44 and RHAMM in human pancreatic cancer cells. *Clin Canc Res.* 1996, 2:1607–18.

2. Adamia S, Maxwell CA, Pilarski LM. Hyaluronan and hyaluronan synthases: potential therapeutic targets in cancer. *Curr Drug Targets Cardiovasc Haematol Disord.* 2005, 5:3-14.

3. Ahrens T, Assmann V, Fieber C, *et al.* CD44 is the principal mediator of hyaluronic-acid-induced melanoma cell proliferation. *J Invest Dermatol.* 2000, 11:93-101.

4. Algar EM, Khromykh T, Smith SI, *et al.* A WT1 antisense oligonucleotide inhibits proliferation and induces apoptosis in myeloid leukemia cell lines. *Oncogene.* 1996, 12:1005-14.

5. Alvarez S, MacGrogan D, Calasanz MJ, *et al.* Frequent gain of chromosome 19 in megakaryoblastic leukemias detected by comparative genomic hybridization. *Genes Chromosomes Cancer.* 2001, 32:285-93.

6. Armstrong JF, Pritchard-Jones K, Bickmore WA, *et al.* The expression of the Wilms' tumor gene, WT1, in the developing mammalian embryo. *Mech Dev.* 1993, 40:85–97.

7. Assmann V, Marshall JF, Fieber C, *et al.* The human hyaluronan receptor RHAMM is expressed as an intracellular protein in breast cancer cells. *J Cell Sci.* 1998, 111:1685–94.

8. Beillard E, Pallisgaard N, van der Velden VH, *et al.* Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR) - a Europe against cancer program. *Leukemia.* 2003, 17:2474-86.

9. Bergmann L, Maurer U, Weidmann E. Wilms tumor gene expression in acute myeloid leukemias. *Leuk Lymphoma.* 1997a, 25:435-43.

10. Bergmann L, Miething C, Maurer U, *et al.* High levels of Wilms' tumor gene (WT1) mRNA in acute myeloid leukemias are associated with a worse long-term outcome. *Blood.* 1997b, 90:1217–25.

11. Boyapati A, Kanbe E, Zhang DE. p53 alterations in myeloid leukemia. *Acta Haematol*. 2004, 111:100-6.

12. Brieger J, Weidmann E, Fenchel K, *et al.* The expression of the Wilms'tumor gene in acute myelocytic leukemias as a possible marker for leukemic blast cells. *Leukemia.* 1994, 8:2138-43.

13. Buganim Y, Rotter V. RHAMM in the complex p53 cell cycle network. *Cell cycle.* 2008, 7:21.

14. Burwell EA, McCarty GP, Simpson LA, *et al.* Isoforms of Wilms' tumor suppressor gene (WT1) have distinct effects on mammary epithelial cellsEffects of WT1 isoforms on breast epithelial cells. *Oncogene*. 2007, 26:3423-30.

15. Call KM, Glaser T, Ito CY, *et al.* Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms tumor locus. *Cell*. 1990, 60:509–20.

16. Cao H, Kanki P, Sankalé JL, *et al.* Cytotoxic T-lymphocyte cross-reactivity among different human immunodeficiency virus type 1 clades: implications for vaccine development. *J Virol.* 1997, 71:8615-23.

17. Casalegno-Garduño R, Schmitt A, Schmitt M. Clinical peptide vaccination trials for leukemia patients. *Expert Rev Vaccines*. 2011, 10:785-99.

18. Casalegno-Garduño R, Schmitt A, Wang X, *et al.* Wilms' tumor 1 as a novel target for immunotherapy of leukemia. *Transplant Proc.* 2010, 42:3309-11.

19. Chen J, Schmitt A, Chen B, *et al.* Nilotinib hampers the proliferation and function of $CD8^+$ T lymphocytes through inhibition of T cell receptor signaling. *J Cell Mol Med.* 2008, 12:2107-18.

20. Chen J, Schmitt A, Giannopoulos K, *et al.* Imatinib impairs the proliferation and function of CD4+CD25+ regulatory T cells in a dose-dependent manner. *Int J Oncol.* 2007, 31:1133-9.

21. Cilloni D, Gottardi E, Messa F, *et al.* Significant correlation between the degree of WT1 expression and the International Prognostic Scoring System Score in patients with myelodysplastic syndromes. *J Clin Oncol.* 2003, 21:1988-95.

22. Cilloni D, Messa F, Arruga F, *et al.* Early prediction of treatment outcome in acute myeloid leukemia by measurement of WT1 transcript levels in peripheral *Blood* samples collected after chemotherapy. *Haematologica.* 2008, 93:921-4.

23. Cilloni D, Renneville A, Hermitte F, *et al.* Real time quatitative polymerase chain reaction detection of minimal residual disease by standardized WT1 assay to enhance risk stratification in acute myeloid leukemia: A European Leukemia Net Study. *J Clin Oncol*. 2009, 27:5195-201.

24. Cilloni D, Saglio G. WT1 as a universal marker for minimal residual disease detection and quantification in myeloid leukemias and in myelodysplastic syndrome. *Acta Haematol.* 2004, 112:79-84.

25. Clark AJ, Dos Santos WG, McCready J, *et al.* Wilms tumor 1 expression in malignant gliomas and correlation of +KTS isoforms with p53 status. *J Neurosurg.* 2007, 107:586-92.

26. Crainie M, Belch AR, Mant MJ, *et al.* Overexpression of the receptor for hyaluronan-mediated motility (RHAMM) characterizes the malignant clone in multiple myeloma: identification of three distinct RHAMM variants. *Blood.* 1999, 93:1684-96.

27. Distler E. *In vitro* generation and characterization of acute myeloid leukemia-reactive $CD8^+$ cytotoxic T-lymphocyte clones from healthy donors. Thesis. 2007. Mainz, Germany.

28. Englert C, Maheswaran S, Garvin AJ, *et al.* Induction of p21 by the Wilms' tumor suppressor gene WT1. *Cancer Res*. 1997, 57:1429-34.

29. Estey E, Döhner H. Acute myeloid leukemia. *Lancet.* 2006, 368:1894-907.

30. Fröhling S, Scholl C, Gilliland DG, Levine RL. Genetics of myeloid malignancies: pathogenetic and clinical implications. *J Clin Oncol*. 2005, 23:6285-95.

31. Gaidzik VI, Schlenk RF, Moschny S, *et al.* Prognostic impact of WT1 mutations in cytogenetically normal acute myeloid leukemia: a study of the German-Austrian AML Study Group. *Blood*. 2009, 113:4505-11.

32. Gaiger A, Carter L, Greinix H, *et al.* WT1-specific serum antibodies in patients with leukemia. *Clin Cancer Res.* 2001, 7 (Suppl 3): 761s–5s.

33. Gao F, Yang CX, Mo W, *et al.* Hyaluronan oligosaccharides are potential stimulators to angiogenesis via RHAMM mediated signal pathway in wound healing. *Clin Invest Med.* 2008, 31:E106-16.

34. Giannopoulos K, Dmoszynska A, Kowal M, *et al.* Peptide vaccination elicits leukemia-associated antigen-specific cytototxic $CD8^+$ T cell-responses in patients with chronic lymphocytic leukemia. *Leukemia.* 2010, 24:798-805.

35. Giles FJ, Keating A, Goldstone AH, *et al.* Acute myeloid leukemia. *Hematology.* 2002, 73-110.

36. Godar S, Weinberg RA. Filling the mosaic of p53 actions: p53 represses RHAMM expression. *Cell Cycle*. 2008, 7:3479.

37. Greiner J, Döhner H, Schmitt M. Cancer vaccines for patients with acute myeloid leukemia (AML) - definition of targets and current clinical protocols (review). *Haematologica.* 2006, 91:1653-61.

38. Greiner J, Guinn B, Döhner H, *et al.* Leukemia-associated antigens (LAAs) are critical for the proliferation of acute myeloid leukemia cells. *Clin Cancer Res.* 2008, 14:1-6.

39. Greiner J, Li L, Ringhoffer M, *et al.* Identification and characterization of epitopes of the receptor for hyaluronic acid mediated motility (RHAMM/CD168) recognized by CD8 positive T cells of HLA-A2 positive patients with acute myeloid leukemia. *Blood.* 2005, 106:938-45.

40. Greiner J, Ringhoffer M, Taniguchi M, *et al.* Characterization of several leukemia associated antigens inducing humoral immune responses in acute and chronic myeloid leukemia (AML/CML). *Int J Cancer.* 2003, 106:224-31.

41. Greiner J, Ringhoffer M, Taniguchi M, *et al.* mRNA expression of leukemia-associated antigens in patients with acute myeloid leukemia for the development of specific immunotherapies*. Int J Cancer*. 2004, 108:704-11.

42. Greiner J, Ringhoffer M, Taniguchi M, *et al.* Receptor for hyaluronan acid-mediated motility (RHAMM) is a new immunogenic tumor-associated antigen overexpressed in acute and chronic myeloid leukemia. *Exp Hematol.* 2002, 30:1029-25.

43. Greiner J, Schmitt A, Giannopoulos K, *et al.* High-dose RHAMM-R3 peptide peptide vaccination for patients with vaccination for patients with acute myeloid leukemia, myelodysplastic syndrome and multiple myeloma. *Haematologica.* 2010, 95:1191-7.

44. Hall CL, Lange LA, Prober DA, *et al.* pp60 (c-src) is required for cell locomotion regulated by the hyaluronan receptor RHAMM. *Oncogene*. 1996, 13:2213–24.

45. Hall CL, Yang B, Yang X, *et al.* Overexpression of the hyaluronan receptor RHAMM is transforming and is also required for H-ras transformation*. Cell.* 1995, 82:19–26

46. Hamilton SR, Fard SF, Paiwand FF, *et al.* The hyaluronan receptors CD44 and Rhamm (CD168) form complexes with ERK1, 2 that sustain high basal motility in breast cancer cells. *J Biol Chem*. 2007, 282:16667-80.

47. Hewitt SM, Saunders GF. Differentially spliced exon 5 of the Wilms' tumor gene WT1 modifies gene function. *Anticancer Res*. 1996, 16:621–6.

48. Horner MJD, Ries LAG. Leukemia. In: Ries LAG, Young JL, Keel GE, *et al.* (eds). SEER Survival Monograph: Cancer Survival Among Adults: U.S. SEER Program, 1988-2001, Patient and Tumor Characteristics. National Cancer Institute, SEER Program. 2007, Chapter 29.

49. Hosen N, Shirakata T, Nishida S, *et al.* The Wilms' tumor gene WT1-GFP knock-in mouse reveals the dynamic regulation of WT1 expression in normal and leukemic hematopoiesis. *Leukemia*. 2007, 21:1783-91.

50. Inoue K, Ogawa H, Yamagami T, *et al.* Long-term follow-up of minimal residual disease in leukemia patients by monitoring WT1 (Wilms tumor gene) expression levels. *Blood.* 1996, 88:2267–78.

51. Inoue K, Sugiyama H, Ogawa H, *et al.* WT1 as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. *Blood.* 1994, 84:3071–79.

52. Inoue K, Tamaki H, Ogawa H *et al.* Wilms' tumor gene (WT1) competes with differentiation-inducing signal in hematopoietic progenitor cells. *Blood.* 1998, 91:2969-76.

53. Ito K, Oji Y, Tatsumi N, *et al.* Antiapoptotic function of 17AA(+) WT1 (Wilms' tumor gene) isoforms on the intrinsic apoptosis pathway. *Oncogene*. 2006, 25:4217-29.

54. Jiang J, Casalegno-Garduno R, Chen H, *et al.* Multifunctional proteins bridge mitosis with motility and cancer with inflammation and arthritis. *Scientific World Journal.* 2010, 10:1244-57.

55. Karnan S, Tsuzuki S, Kiyoi H, *et al.* Genomewide array-based comparative genomic hybridization analysis of acute promyelocytic leukemia. *Genes Chromosomes Cancer.* 2006, 45:420-5.

56. Keilholz U, Letsch A, Busse A, *et al.* A clinical and immunologic phase 2 trial of Wilms tumor gene product 1 (WT-1) peptide vaccination in patients with AML and MDS. *Blood.* 2009, 113:6541-8.

57. Krug U, Ganser A, Koeffler HP. Tumor suppressor genes in normal and malignant hematopoiesis. *Oncogene*. 2002, 21:3475–95.

58. Kuzushima K, Sun R, van Bleek GM, *et al.* The role of self peptides in the allogeneic cross-reactivity of CTLs. *J Immunol.* 1995, 155:594-601.

59. Lai JL, Preudhomme C, Zandecki M, *et al.* Myelodysplastic syndromes and acute myeloid leukemia with 17p deletion. An entity characterized by specific dysgranulopoiesis and a high incidence of p53 mutations. *Leukemia.* 1995, 9:370–81.

60. Laity JH, Dyson HJ, Wright PE. Molecular basis for modulation of biological function by alternate splicing of the Wilms' tumor suppressor protein. *Proc Natl Acad Sci U S A.* 2000, 97:11932-5.

61. Lapidot T, Kollet O. The essential roles of the chemokine SDF-1 and its receptor CXCR4 in human stem cell homing and repopulation of transplanted immune-deficient NOD/SCID and NOD/SCID/B2m(null) mice. *Leukemia*. 2002, 16:1992–2003.

62. Li H, Guo L, Li JW, *et al.* Expression of hyaluronan receptors CD44 and RHAMM in stomach cancers: relevance with tumour progression. *Int J Oncol.* 2000, 17:927–32.

63. Li H, Oka Y, Tsuboi A, *et al.* The lck promoter-driven expression of the Wilms tumor gene WT1 blocks intrathymic differentiation of T-lineage cells. *Int J Hematol.* 2003, 77:463-70.

64. Li L, Reinhardt P, Schmitt A, *et al.* Dendritic cells generated from acute myeloid leukemia (AML) blasts maintain the expression of immunogenic leukemia associated antigens. *Cancer Immunol Immunother.* 2005, 54:685-93.

65. Line A, Slucka Z, Stengrevics A, *et al.* Characterisation of tumour associated antigens in colon cancer. *Cancer Immunol Immunother.* 2002, 51:574-82.

66. Loeb DM, Summers JL, Burwell EA, *et al.* An isoform of the Wilms' tumor suppressor gene potentiates granulocytic differentiation. *Leukemia*. 2003, 17:965-71.

67. Maheswaran S, Park S, Bernard A, *et al.* Physical and functional interaction between WT1 and p53 proteins. *Proc Natl Acad Sci U S A.* 1993, 90:5100-4.

68. Mailänder V, Scheibenbogen C, Thiel E, *et al.* Complete remission in a patient with recurrent acute myeloid leukemia induced by vaccination with WT-1 peptide in the absence of hematological or renal toxicity. *Leukemia.* 2004, 18:165-6.

69. Mani J, Schmitt A, Casalegno-Garduño R, *et al.* Phenotype and functional cross-reactivity in JC and BK polyoma virus peptide specific T cells. *Onkologie.* 2011, 34 (Suppl. 6) Abs. V729. (Abstract).

70. Maslak PG, Dao T, Krug LM, *et al.* Vaccination with synthetic analog peptides derived from WT1 oncoprotein induces T-cell responses in patients with complete remission from acute myeloid leukemia. *Blood.* 2010, 116:171-179.

71. Mason RD, Bowmer MI, Howley CM, Grant MD. Cross-reactive cytotoxic T lymphocytes against human immunodeficiency virus type 1 protease and gamma interferon-inducible protein 30. *J Virol.* 2005, 79:5529-36.

72. Maurer U, Brieger J, Weidmann E, *et al*. The Wilms' tumor gene is expressed in a subset of CD34+ progenitors and downregulated early in the course of differentiation in vitro. *Exp Hematol.* 1997, 25:945–50.

73. Maxwell CA, Keats JJ, Crainie M, *et al.* RHAMM is a centrosomal protein that interacts with dynein and maintains spindle pole stability. *Mol Biol Cell.* 2003, 14:2262-76.

74. Maxwell CA, McCarthy J, Turley E. Cell-surface and mitotic-spindle RHAMM: moonlighting or dual oncogenic functions? *J Cell Sci.* 2008, 121:925-32.

75. Maxwell CA, Rasmussen E, Zhan F, *et al.* RHAMM expression and isoform balance predict aggressive disease and poor survival in multiple myeloma. *Blood.* 2004, 104:1151-8.

76. McQueen T, Konopleva M, Andreeff M. Activity of targeted molecular therapeutics against primary AML cells: putative role of the bone marrow microenvironment. *Blood.* 2005, 106 (suppl 11): 2304 (Abs).

77. Menssen HD, Renkl HJ, Maurer J, *et al.* Presence of the Wilms' tumor gene (wtl) transcripts and the WTI nuclear protein in the majority of human acute leukemias. *Leukemia.* 1995, 9:1060-7.

78. Misko IS, Cross SM, Khanna R, *et al.* Crossreactive recognition of viral, self, and bacterial peptide ligands by human class I-restricted cytotoxic T lymphocyte clonotypes: implications for molecular mimicry in autoimmune disease. *Proc Natl Acad Sci U S A.* 1999, 96:2279-84.

79. Miwa H, Beran M, Saunders GF. Expression of the Wilms' tumor gene (WT1) in human leukemias. *Leukemia.* 1992, 6:405–9.

80. Mohapatra S, Yang X, Wright JA, *et al.* Soluble hyaluronan receptor RHAMM induces mitotic arrest by suppressing Cdc2 and cyclin B1 expression. *J Exp Med.* 1996, 183:1663-8.

81. Nagy JI, Hacking J, Frankenstein UN, Turley EA. Requirement of the hyaluronan receptor RHAMM in primary neurons and neuronal cell lines. *J Neurosci.* 1995, 15:241–52.

82. Naor D, Nedvetzki S, Walmsley M, *et al.* CD44 involvement in autoimmune inflammations: the lesson to be learned from CD44-targeting by antibody or from knockout mice. *Ann N Y Acad Sci.* 2007, 1110:233-47.

83. Nara N. The role of cytokines in the proliferation of leukemia cells in acute myelogenous leukemia. *Rinsho Byori.* 1993, 41:1289-95.

84. Narita M, Masuko M, Kurasaki T, *et al.* WT1 peptide vaccination in combination with imatinib therapy for a patient with CML in the chronic phase. *Int J Med Sci.* 2010, 7:72-81.

85. Nedvetzki S, Gonen E, Assayag N, *et al.* RHAMM, a receptor for hyaluronan-mediated motility, compensates for CD44 in inflamed CD44-knockout mice: a different interpretation of redundancy. *Proc Natl Acad Sci U S A*. 2004, 101:18081-6.

86. Nicoli P, Defilippi I, Carturan S, *et al.* Detection of humoral immune responses against WT1 antigen in patients affected by different hematological malignancies. *Acta Haematol.* 2008, 120:47-50.

87. Nowakowska-Kopera A, Sacha T, Florek I, *et al.* Wilms' tumor gene 1 expression analysis by real-time quantitative polymerase chain reaction for monitoring of minimal residual disease in acute leukemia*. Leuk Lymphoma.* 2009, 50:1326–32.

88. Oji Y, Oka Y, Nishida S, *et al.* WT1 peptide vaccine induces reduction in minimal residual disease in an Imatinib-treated CML patient. *Eur J Haematol.* 2010, 85:358–360.

89. Oka Y, Elisseeva OA, Tsuboi A, *et al.* Human cytotoxic T-lymphocyte responses specific for peptides of the wild-type Wilms' tumor gene (WT1) product*. Immunogenetics.* 2000b, 51:99-107.

90. Oka Y, Tsuboi A, Taguchi T *et al.* Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression. *Proc Natl Acad Sci.* 2004, 101:13885-90.

91. Oka Y, Udaka K, Tsuboi A, *et al.* Cancer immunotherapy targeting Wilms' tumor gene WT1 product. *J Immunol.* 2000a, 164:1873–80.

92. Osaka M, Koami K, Sugiyama T. WT1 contributes to leukemogenesis: expression patterns in 7,12-dimethylbenz[a]anthracene (DMBA)-induced leukemia. *Int J Cancer*. 1997, 72:696–9.

93. Prokocimer M, Peller S. Cytoplasmic sequestration of wild-type p53 in a patient with therapy-related resistant AML: first report. *Med Oncol.* 2011. [Epub ahead of print].

94. Prokocimer M, Shaklai M, Bassat HB, *et al.* Expression of p53 in human leukemia and lymphoma. *Blood.* 1986, 68: 113-8.

95. Pujana MA, Han JD, Starita LM, *et al.* Network modeling links breast cancer susceptibility and centrosome dysfunction. *Nat Genet.* 2007, 39:1338-49.

96. Rein DT, Roehrig K, Schöndorf T, *et al.* Expression of the hyaluronan receptor RHAMM in endometrial carcinomas suggests a role in tumour progression and metastasis. *J Cancer Res Clin Oncol*. 2003, 129:161-4.

97. Rezvani K. PR1 vaccination in myeloid malignancies. *Expert Rev Vaccines.* 2008, 7:867-5.

98. Samuel SK, Hurta RA, Spearman MA, *et al.* TGF-beta 1 stimulation of cell locomotion utilizes the hyaluronan receptor RHAMM and hyaluronan. *J Cell Biol.* 1993, 123:749-58.

99. Schmid D, Heinze G, Linnerth B, *et al.* Prognostic significance of WT-1 gene expression at diagnosis in adult de novo acute myeloid leukemia. *Leukemia.* 1997, 11:639-43.

100. Schmitt M, Neubauer A, Greiner J, *et al.* Spreading of acute myeloid leukemia cells by trafficking along the peripheral outflow pathway of cerebrospinal fluid. *Anticancer Res*. 2011, 31:2343-5.

101. Schmitt M, Schmitt A, Rojewski M, *et al.* RHAMM-R3 peptide vaccination in patients with acute myeloid leukemia, myelodysplastic syndrome and multiple myeloma elicits immunological and clinical responses. *Blood.* 2008, 111:1357-65.

102. Seggewiss R, Loré K, Greiner E, *et al.* Imatinib inhibits T-cell receptor-mediated T-cell proliferation and activation in a dose-dependent manner. *Blood.* 2005, 105:2473-9.

103. Slevin M, Krupinski J, Gaffney J, *et al.* Hyaluronan-mediated angiogenesis in vascular disease: uncovering RHAMM and CD44 receptor signaling pathways. *Matrix Biol.* 2007, 26:58-68.

104. Sohr S, Engeland K. RHAMM is differentially expressed in the cell cycle and downregulated by the tumor suppressor p53. *Cell Cycle.* 2008, 7:3448-60.

105. Spicer AP, Roller ML, Camper SA, *et al.* The human and mouse receptors for hyaluronan-mediated motility, RHAMM, genes (HMMR) map to human chromosome 5q33.2-qter and mouse chromosome 11. *Genomics.* 1995, 30:115-7.

106. Stretch JR, Gatter KC, Ralfkiaer E, *et al.* Expression of mutant p53 in melanoma. *Cancer Res.* 1991, 51:5976-9.

107. Sugiyama H. Wilms tumor gene (WT1) as a new marker for the detection of minimal residual disease in leukemia. *Leuk Lymphoma.* 1998, 30:55–61.

108. Tallman MS, Gilliland DG, Rowe JM. Drug therapy of acute myeloid leukemia. *Blood*. 2005, 106: 1154–63.

109. Tatsumi N, Oji Y, Tsuji N, *et al.* Wilms' tumor gene WT1-shRNA as a potent apoptosis-inducing agent for solid tumors. *Int J Oncol.* 2008, 32:701-11.

110. Teder P, Bergh J, Heldin P. Functional hyaluronan receptors are expressed on a squamous cell lung carcinoma cell line but not on other lung carcinoma cell lines. *Cancer Res.* 1995, 55:3908-14.

111. Thoene S, Rawat VP, Heilmeier B, *et al.* The homeobox gene CDX2 is aberrantly expressed and associated with an inferior prognosis in patients with acute lymphoblastic leukemia. *Leukemia*. 2009, 23:649-55.

112. Till KJ, Zuzel M, Cawley JC. The role of hyaluronan and interleukin 8 in the migration of chronic lymphocytic leukemia cells within lymphoreticular tissues. *Cancer Res*. 1999, 59:4419-26.

113. Tolg C, Hamilton SR, Nakrieko KA, *et al.* Rhamm–/– fibroblasts are defective in CD44-mediated ERK1,2 motogenic signaling, leading to defective skin wound repair. *J Cell Biol.* 2006, 175:1017-28.

114. Turley EA, Harrison A. RHAMM a member of the hyaladherins. Glycoforum. 1999

115. Turley EA, Noble PW, Bourguignon LY. Signaling properties of hyaluronan receptors. *J Biol Chem*. 2002, 277:4589-92.

116. Turley EA. Purification of a hyaluronate-binding protein fraction that modifies cell social behavior. *Biochem Biophys Res Commun* 1982, 108:1016-24.

117. Tzankov A, Strasser U, Dirnhofer S, *et al.* In situ RHAMM protein expression in acute myeloid leukemia blasts suggests poor overall survival. *Ann Hematol.* 2011, 90:901-9.

118. Wagner N, Wagner KD, Hammes A, *et al.* A splice variant of the Wilms' tumour suppressor Wt1 is required for normal development of the olfactory system. *Development.* 2005, 132:1327-36.

119. Wang C, Thor AD, Moore DH 2nd, *et al.* The overexpression of RHAMM, a hyaluronan-binding protein that regulates ras signaling, correlates with overexpression of mitogen-activated protein kinase and is a significant parameter in breast cancer progression. *Clin. Cancer Res*. 1998, 4:567-76.

120. Wang X, Schmitt A, Chen B, *et al*. Streptamer-based selection of WT1-specific $CD8^+$ T cells for specific donor lymphocyte infusions. *Exp Hematol.* 2010, 38:1066-73.

121. Weber G, Karbach J, Kuçi S, *et al.* WT1 peptide-specific T cells generated from peripheral blood of healthy donors: possible implications for adoptive immunotherapy after allogeneic stem cell transplantation. *Leukemia*. 2009, 23:1634-42.

122. Zügel U, Schoel B, Yamamoto S, *et al.* Crossrecognition by CD8 T cell receptor alpha beta cytotoxic T lymphocytes of peptides in the self and the mycobacterial hsp60 which share intermediate sequence homology. *Eur J Immunol.* 1995, 25:451-8.

123. http://seer.cancer.gov/statfacts/html/leuks.html#incidence-mortality

6 SUPPLEMENTAL MATERIAL

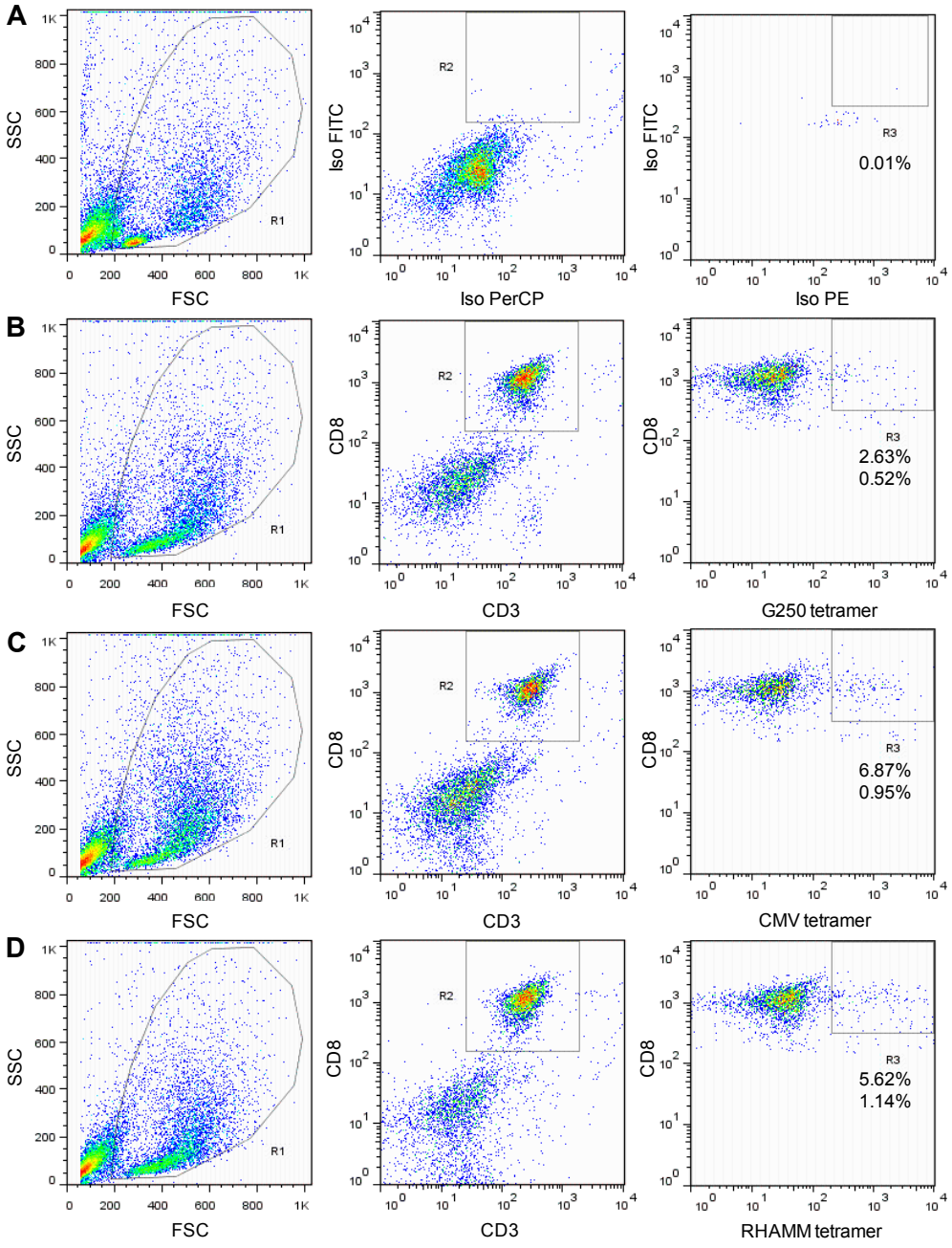

Figure S1. RHAMM-specific T cells in a RHAMM$^+$ patient (Pat. No. 5) at diagnosis (April 2010). Reported frequencies correspond to gate CD3$^+$ CD8$^+$ (upper number, R3), and from the total amount of cells. **A.** Isotype control, **B.** Non-peptide control, stained with irrelevant tetramer, **C.** Positive control, CTLs were stimulated with CMVpp65 peptide, **D.** CTLs were stimulated with RHAMM peptide. **C-D.** CTLs were stained with respective tetramers.

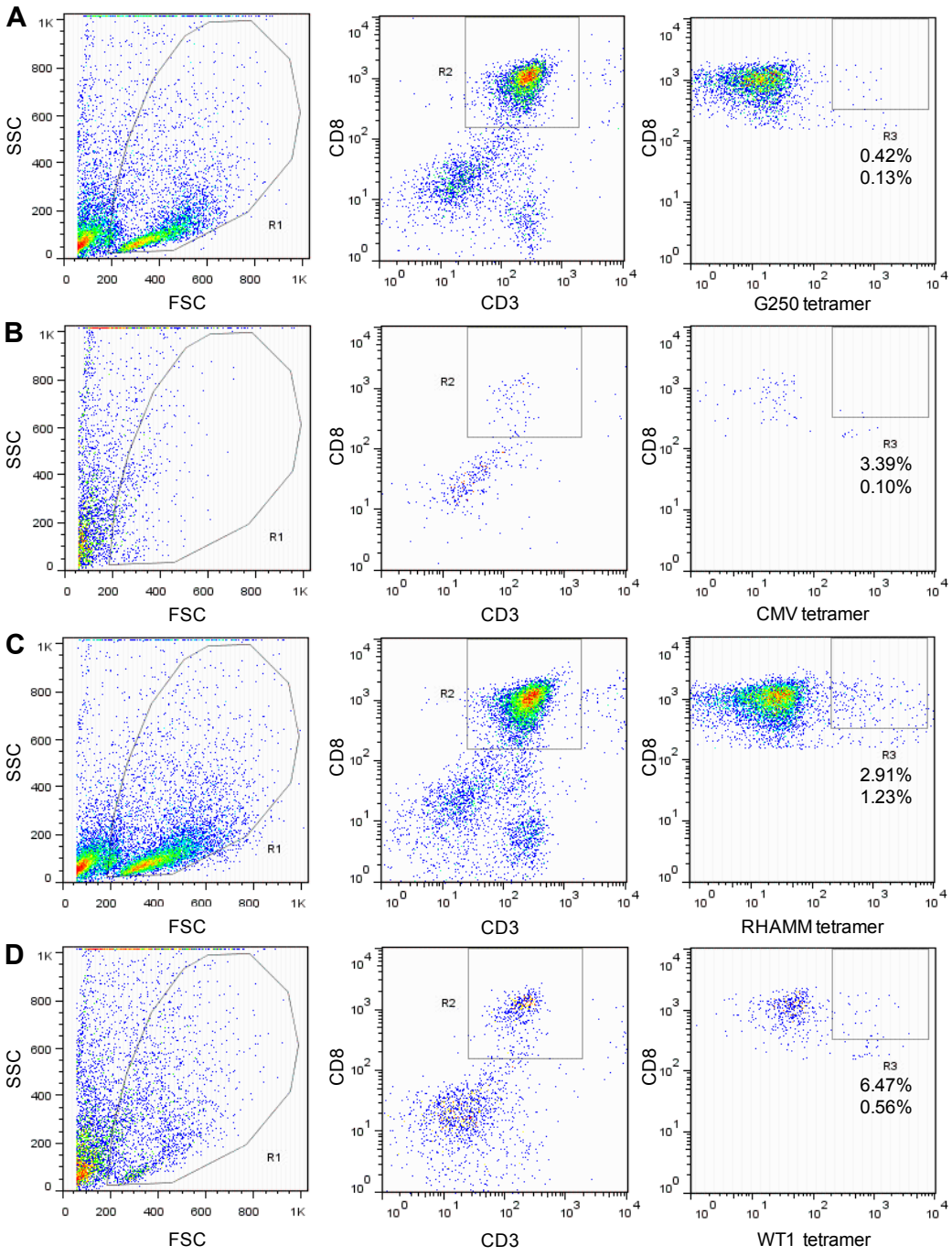

Figure S2. RHAMM-specific T cells in a RHAMM$^+$ patient (Pat. No. 5) during CR (June 2010). Reported frequencies correspond to gate CD3$^+$ CD8$^+$ (upper number, R3), and from the total amount of cells. Isotype control is shown in Figure S1. **A.** Non-peptide control, stained with irrelevant tetramer, **B.** Positive control, CTLs were stimulated with CMVpp65 peptide, **C.** CTLs were stimulated with RHAMM peptide, **D.** CTLs were stimulated with WT1 peptide. **B-D.** CTLs were stained with respective tetramers.

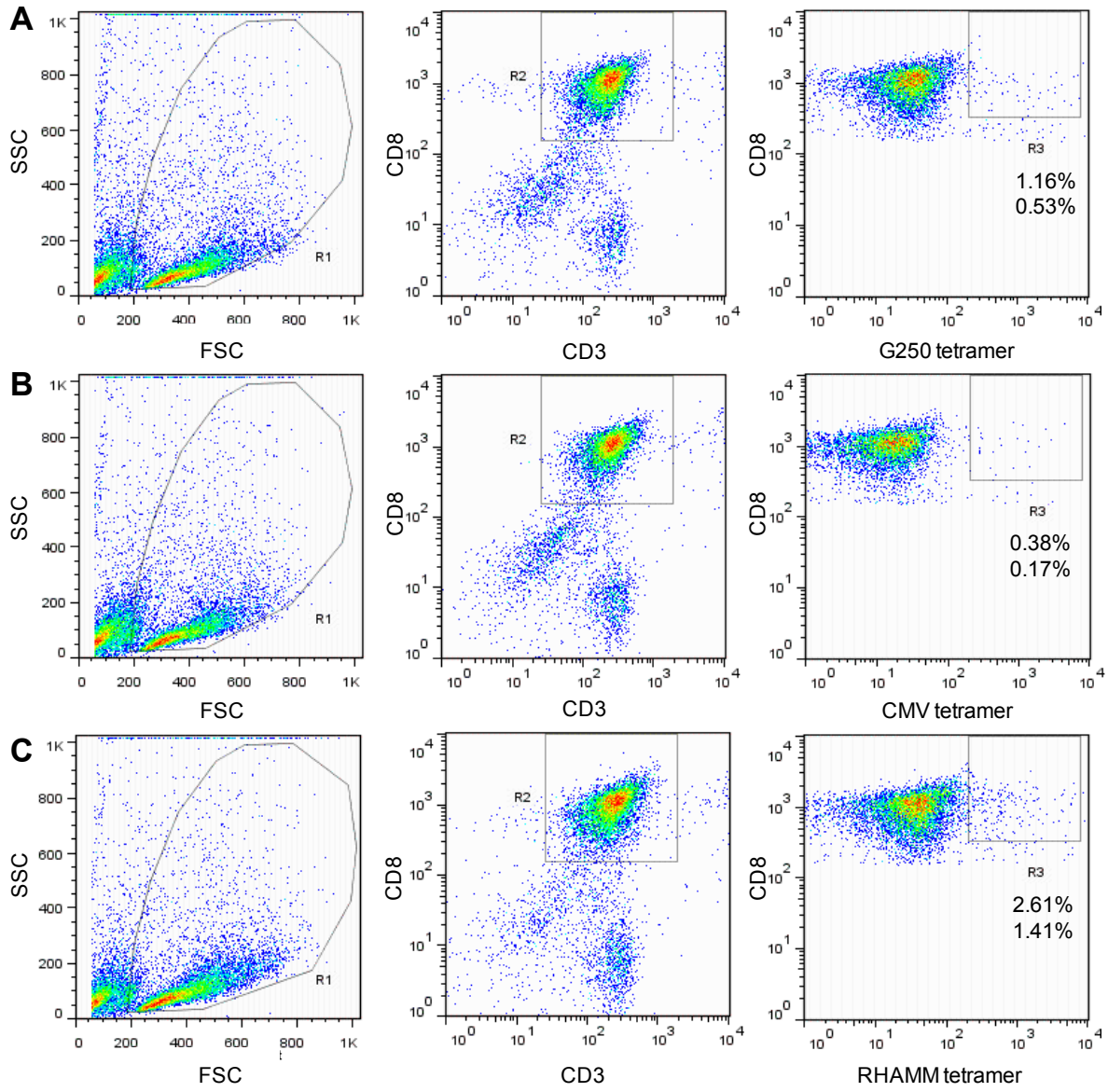

Figure S3. RHAMM-specific T cells in a RHAMM$^+$ patient (Pat. No. 5) during CR (July 2010). Reported frequencies correspond to gate CD3$^+$ CD8$^+$ (upper number, R3), and from the total amount of cells. Isotype control is shown in Figure S1. **A.** Non-peptide control, stained with irrelevant tetramer, **B.** Positive control, CTLs were stimulated with CMVpp65 peptide, **C.** CTLs were stimulated with RHAMM peptide. **B-C.** CTLs were stained with respective tetramers.

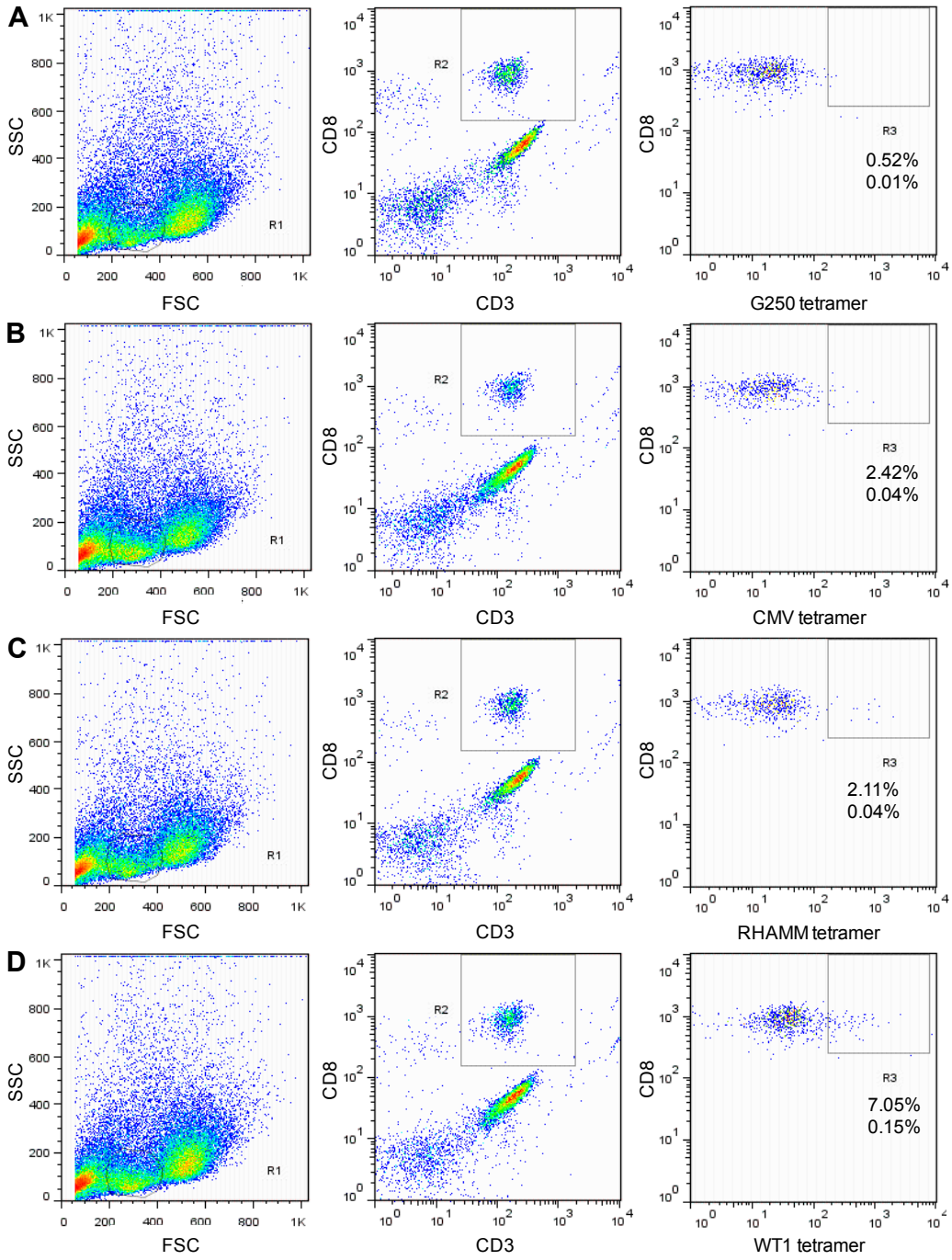

Figure S4. RHAMM-specific T cells in a RHAMM$^+$ patient (Pat. No. 5) at relapse (February 2011). Reported frequencies correspond to gate CD3$^+$ CD8$^+$ (upper number, R3), and from the total amount of cells. Isotype control is shown in Figure S1. **A**. Non-peptide control, stained with irrelevant tetramer, **B**. Positive control, CTLs were stimulated with CMVpp65 peptide, **C**. CTLs were stimulated with RHAMM peptide, **D**. CTLs were stimulated with WT1 peptide. **B-D**. CTLs were stained with respective tetramers.

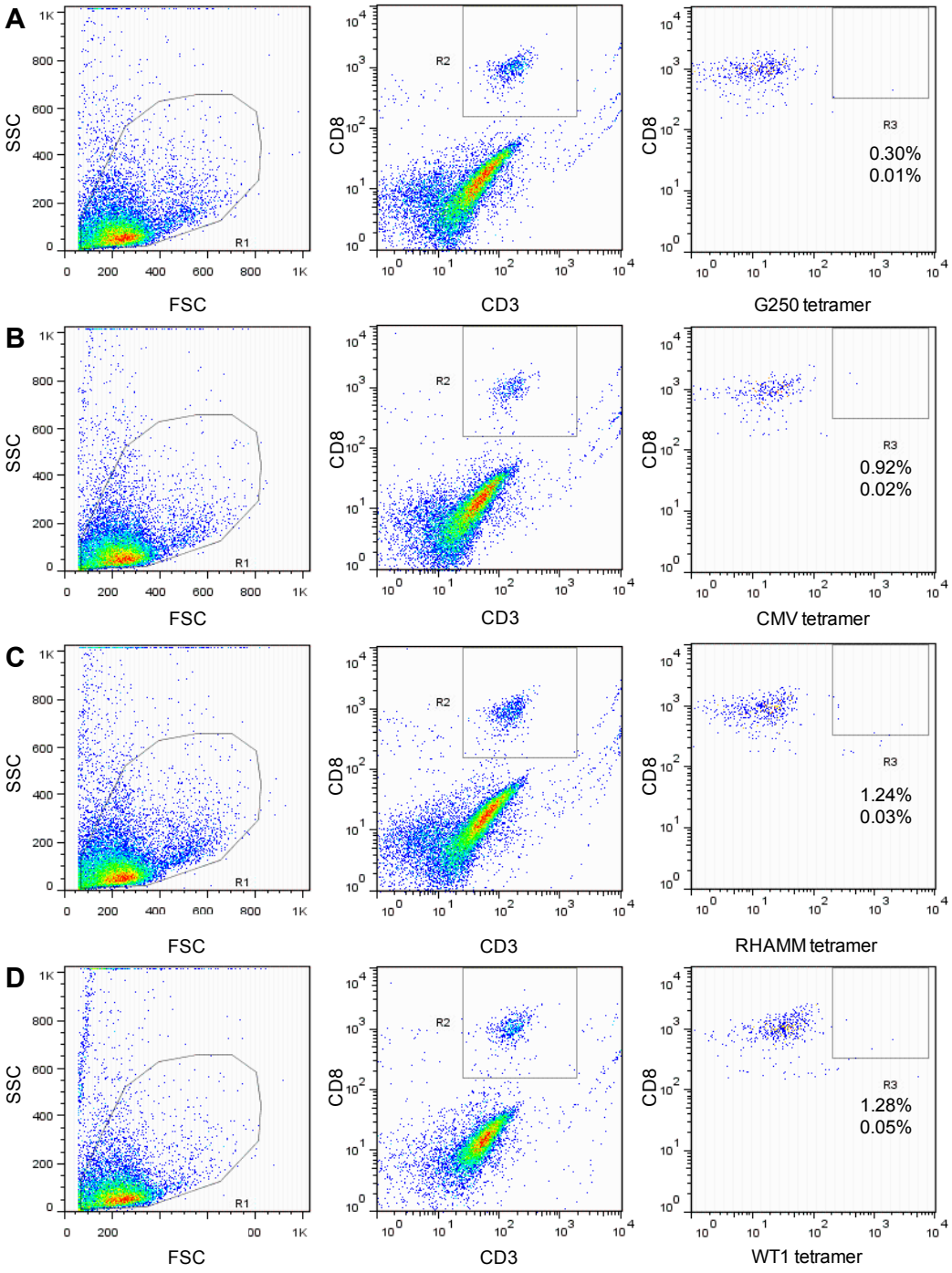

Figure S5. RHAMM-specific T cells in a RHAMM$^+$ patient (Pat. No. 5) at relapse (March 2011). Reported frequencies correspond to gate CD3$^+$ CD8$^+$ (upper number, R3), and from the total amount of cells. Isotype control is shown in Figure S1. **A**. Non-peptide control, stained with irrelevant tetramer, **B**. Positive control, CTLs were stimulated with CMVpp65 peptide, **C**. CTLs were stimulated with RHAMM peptide, **D**. CTLs were stimulated with WT1 peptide. **B-D**. CTLs were stained with respective tetramer.

6.1 SUPPLEMENT 1. BUFFERS FOR IFN-γ ELISPOT

1. Coating buffer for IFNγ

   318 mg Na_2CO_3, 580 mg $NaHCO_3$, 49 mg NaN_3, in 100 ml H_2O.

   0.1 M, pH 9.6

   Sterile filtration (via filter or ejector pump)

2. Substrate buffer for IFNγ

   0.1 M TRIS (6.05g/500ml), 0.1 M NaCl (2.9g/500ml), 5 mM $MgCl_2$ (237mg/500ml)

   pH 9.5

   Sterile filtration via filter or ejector pump

6.2 SUPPLEMENT 2. EQUIPMENT

Autoclave	WEBECO GmbH, Germany
24-well tissue culture plate (Flat bottom)	BD Biosciences, New Jersey, USA
-80°C Ultra low temperature freezer	SANYO Electric Biomedical Co. Ltd, Japan
96-well tissue culture plate (U-bottom)	BD Biosciences, New Jersey, USA
BD FACScan TM Flow Cytometry	BD Biosciences, New Jersey, USA
Combitips plus (1.25 ml, 2.5 ml, 5 ml, 10 ml, 12.5 ml, 25 ml)	Eppendorf Ag, Hamburg, Germany
CS-15R Centrifuge	Beckman Biotechnology, Germany
CS-6R Centrifuge	Beckman Biotechnology, Germany
Ep T.I.P. S Reloads 0.1-20 µl, 2-200 µl, 50-1000 µl,	Eppendorf AG, Hamburg, Germany
Gloves	VWR International GmbH, Darmstadt, Germany
Hemocytometer Chamber	Optik Labor, Berlin, Germany
Inverse microscope	Carl Zeiss, TELAVAC31, Germany
Light microscope	Carl-Zeiss, Germany
Liquid nitrogen tank	Model 8038 S/N 14830.59 Forma Scientific Inc. Germany
Magnetic stirrer	Ikamag TRC, Renner GmbH, Ludwigshafen, Germany
MS columns	Miltenyi Biotec, Bergisch Gladbach, Germany
MACS Pre-Separation Filters	Miltenyi Biotec, Bergisch Gladbach,

	Germany
Non pyrogenic serologic pipettes (2 ml, 5 ml, 10 ml, 25 ml, 50 ml)	Corning Incorporation, New York, USA
Pipette Boy (IBS Pipetboy acu)	Integra Biosciences AG, Chur, Switzerland
Polypropylene Conical Centrifuge Tubes, 15 ml	Becton and Dickinson labware, NJ, U.S.A
Polypropylene Conical Centrifuge Tubes, 50 ml	Becton and Dickinson labware, NJ, U.S.A
Power Pac TM HC Power Supply	Bio-Rad, USA
Refrigerator	Liebherr, Ochsenhausen, Gemany
Sterile bench (Laminar flow cabinet)	TYP. HS 18/2, Heraeus instruments, Germany
Super MACS ^{TM} separator	Miltenyi Biotec, Bergisch Gladbach, Germany
Thermomixer Compact	Termon Eppendorf, Germany
TWIN Power Calculator SHAPP	China
Incubator Holder	Heraeus, Germany
Vortex-2^{TM} genie	Model G-560E, Scientific Industries, Inc. Bohemia, NY, U.S.A
Water bath	GFL, Burgwedel, Germany

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