THE ASSOCIATION OF GENETIC FACTORS WITH COMPLICATIONS AND OUTCOME OF ALLOGENEIC HAEMATOPOIETIC STEM CELL TRANSPLANTATION

PhD thesis

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1. THE LIST OF ABBREVIATIONS

ADAMTS13	a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13
A	adenine
aGvHD	acute graft-versus-host disease
ALL	acute lymphoblastic leukaemia
Allo-HSCT	allogeneic haematopoietic stem cell transplantation
AML	acute myeloid leukaemia
APC	antigen presenting cell
ATG	anti-thymocyte globulin
BM	bone marrow
BMT	bone marrow transplantation
BMT CTN	Blood and Marrow Transplant Clinical Trials Network
BSA	body surface area
BU	busulfan
C	cytosine
CCR9	C-C motif chemokine receptor 9
CD	cluster of differentiation
CD40L	cluster of differentiation 40 ligand
cGvHD	chronic graft-versus-host disease
CI	confidence interval
CMV	cytomegalovirus
CNI	calcineurin inhibitor
CR	complete remission
CSA	cyclosporine
CTLA-4	cytotoxic T-lymphocyte associated protein 4
CY	cyclophosphamide
D	donor
D0	non-carrier donor
D1	carrier donor
DFS	disease-free survival
DLI	donor lymphocyte infusion

DNA	deoxyribonucleic acid
EBMT	European Society for Blood and Marrow Transplantation
EBV	Epstein-Barr virus
FLU	fludarabine
FU	follow up
G	grade
GI	gastrointestinal
Gu	guanine
GvL	graft-versus-leukaemia
GvHD	graft-versus-host disease
Gy	Gray
HLA	human leucocyte antigen
HPF	high power field
HR	hazard ratio
HSCT	haematopoietic stem cell transplantation
IBD	inflammatory bowel disease
IFNG	interferon gamma
IFNR	interferon gamma receptor
IL	interleukin
IL-2RA	Interleukin-2 receptor subunit alpha
IL-7RA	Interleukin-7 receptor subunit alpha
IL-10RB	interleukin-10 receptor subunit beta
IL-23R	interleukin-23 receptor
INSL4, 6	insulin-like 4, 6 genes
JAK	Janus kinase
LDH	lactate dehydrogenase
MAC	myeloablative conditioning
MEL	melphalan
MHC	major histocompatibility complex
MM	multiple myeloma
MMF	mycophenolate mofetil
MPL	myeloproliferative leukaemia virus oncogene

MPN	myeloproliferative neoplasm
MRD	matched related donor
MUD	matched unrelated donor
mTOR	mammalian target of rapamycin
MTX	methotrexate
N	number
NMDP	National Marrow Donor Program
NRM	non-relapse mortality
OR	odds ratio
OS	overall survival
P	probability value
PCR	polymerase chain reaction
PB	peripheral blood
PBSC	peripheral blood stem cell
PBSCT	peripheral blood stem cell transplantation
PTLD	post-transplant lymphoproliferative disorder
R	recipient
R0	non-carrier recipient
R1	carrier recipient
R0D0	non-carrier recipient and donor
R0D1	non-carrier recipient and carrier donor
R1D0	carrier recipient and non-carrier donor
R1D1	carrier recipient and donor
RCT	randomised controlled trial
Ref	reference
RFS	relapse-free survival
RIC	reduced intensity conditioning
RNA	ribonucleic acid
SIR	sirolimus
SNP	single nucleotide polymorphism
SOS	sinusoidal obstruction syndrome
SSOP	sequence-specific oligonucleotide probe

SSP	sequence-specific primer
STAT	signal transducer and activator of transcription
ST2	suppression of tumorigenicity 2
T	thymine
TAC	tacrolimus
TA-TMA	transplantation-associated thrombotic microangiopathy
TBI	total body irradiation
TGFB1	transforming growth factor beta 1
Th	helper T cell
TMA	thrombotic microangiopathy
TNFA	tumour necrosis factor alpha
TNFB	tumour necrosis factor beta
TNFR1	tumour necrosis factor receptor 1
TNFRII	tumour necrosis factor receptor type 2 gene
Treg	regulatory T cell
TPE	therapeutic plasma exchange
TRM	transplant-related mortality
TTP	thrombotic thrombocytopenic purpura
VEGF	vascular endothelial growth factor
VOD	veno-occlusive disease
VP16	etoposide

2. INTRODUCTION

Since its first implementation in the 1950s, allogeneic haematopoietic stem cell transplantation (allo-HSCT) has become the most widely used cellular immunotherapy (1, 2). Allo-HSCT provides the only curative treatment for several haemato-oncological, immunological and hereditary conditions (3). As such allo-HSCT is a life-saving procedure, which can deliver long term survival in conditions where the prognosis would be very poor without the intervention. In adulthood allo-HSCT is far most frequently implemented in malignant diseases (4). In cancerous conditions the success of the procedure greatly relies on the development of donor originated graft-versus-leukaemia (GvL) effect, which can result in full eradication of the underlying disease (5). This explains why allo-HSCT is categorised as immunotherapy. The road to enable that allo-HSCT could develop to a well-established, routine treatment has led through several steps including the identification that haematopoietic tissues are among the most radiation-sensitive ones and the recognition of the human leucocyte antigen (HLA) system (6). The expanding knowledge on the implications of the HLA genes on transplant immunology very significantly improved donor selection methods and contributed greatly to improved survival (7). Similarly the achievements of supportive care, such as effective methods for screening of viral reactivations, better antimicrobial treatment, nutritional support and the advances of intensive care in this special setting enabled the progress of the field (8, 9). Following the introduction of less intense transplant techniques the procedure has become safely deliverable to older and less fit patients, which is a huge achievement giving that the incidence of many haematological malignancies increases by age (10). Despite the gradually improving outcome, allo-HSCT remains a high risk procedure with the potential to induce many acute and chronic complications, which can all be potentially like-threatening or result in poor quality of life and permanent disability (11). Infections, acute and chronic graft-versus-host disease (GvHD) and organ toxicities represent the major complications of the procedure (11, 8). Enormous research effort has been put into the identification of factors that could predict outcome. In this work we report our findings in association with two severe complications of the procedure, transplantation-associated thrombotic microangiopathy (TA-TMA) and acute graft-versus-host disease (aGvHD).

2.1. OVERVIEW OF ALLOGENEIC HAEMATOPOIETIC STEM CELL TRANSPLANTATION

2.1.1. The development of allogeneic HSCT

2.1.1.1. First therapeutic application of bone marrow transplantation

Haematopoietic stem cell transplantation (HSCT) refers to a procedure where haematopoietic stem cells are administered to a given recipient with the aim to replace the lymphohaematopoietic system (3). The procedure is categorised as autologous in case the stem cells originate from the recipient, syngeneic if cells come from an identical twin or allogeneic if the cells are transplanted from a non-identical twin related or matched unrelated donor (MUD) (3). This work focuses on the latter form of transplantation, the allogeneic HSCT. In 2007 the New England Journal of Medicine published an editorial in honour of Dr Thomas on the 50th anniversary of his first publication about the use of a radically new approach for the treatment of cancer, intravenous infusion of bone marrow (BM) to patients following radiation and chemotherapy (12, 2). His experimental strategy was primed by animal studies showing that mice can survive lethal radiation if their spleen or BM is protected from radiation and that the destroyed BM may be repopulated by intravenous infusion of donor BM cells (13-16). In the first human study none of the six patients survived beyond day 100, but taking into consideration that in those days knowledge was extremely limited on histocompatibility and immunosuppression, this is not surprising (2). Following the recognition of the importance of recipient/donor matching in canine models and that methods became available for HLA-typing the human studies were reiterated. His perseverance and commitment paid off and his group reported the first successful allo-HSCT for aplastic anaemia in 1972 followed by many more publications in the upcoming years on long term cure for leukaemia with this approach (17-21). Dr Thomas was awarded a joint Nobel Prize in 1990 for "his discoveries concerning cell transplantation in the treatment of human disease". The award was well-deserved giving that his pioneering work led to the cure of thousands of otherwise incurable patients and the number of individuals benefiting from this modality will further increase in the future (12). The commemorative publication summarised the advances achieved in the first 50 years of transplantation as showed on **Figure 1.** (12). Major

achievements were made via the development of histocompatibility testing, the introduction of prophylactic immunosuppression for the prevention of graft rejection and GvHD, the use of alternative donor sources and the introduction of reduced intensity conditioning (RIC) regimens enabling broader access to the procedure for older and infirm patients.

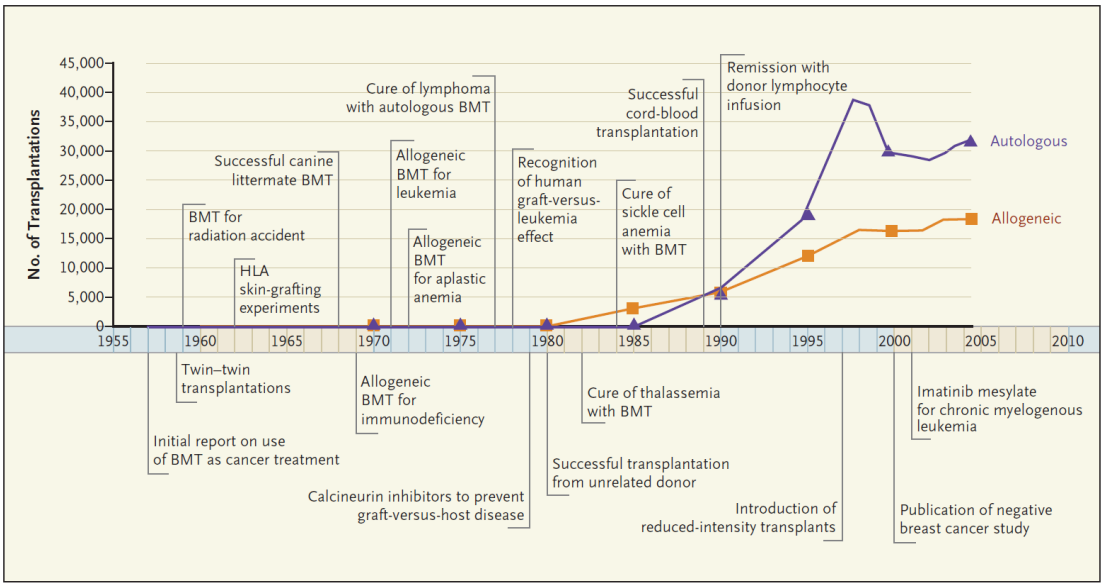

Figure 1. Milestones and advances in the HSCT field and numbers of autologous and allogeneic transplants in the first 50 years of transplantation history between 1957 and 2006. Adapted from Appelbaum, 2007 (12).

2.1.1.2. Trends worldwide and in Hungary

Allogeneic HSCT has by now become a standard of care for numerous haematological and other (oncological, metabolic, autoimmune) conditions (3). The European Society for Blood and Marrow Transplantation (EBMT) is the major European organisation that overlook the transplantation activity in European and some affiliated countries. EBMT regularly report outcome data, publish recommendations and facilitate training events for various specialties involved in the field. EBMT have been collecting data on transplant activity for decades and the first transplantation activity survey was conducted in 1990 (1). Since then a survey has been performed yearly and the data have been annually reported in the journal of Bone Marrow Transplantation. In 2012 EBMT published a summary for the first 20 years of the survey experience within the period of 1990-2010,

and highlighted the trends observed within two decades of practice (1). These included the gradually rising number of transplants (almost ten times more procedures in 2010 compared with 1990) and the increasing age of transplant recipients following the introduction of reduced intensity conditioning. There was a shift in indications (less cases with chronic myeloid leukaemia [CML] due to the introduction of tyrosine kinase inhibitors, more transplants for myelodysplastic syndrome [MDS] and lymphoma). The number of MUD transplants has been quickly expanding and since 2008 has outnumbered sibling transplants (1).

As outlined in the last EBMT survey published in 2016 Hungary was among the countries showing significant (slightly more than 100%) increase in allo-HSCT activity between 2004 and 2014 (4). The above trends the transplant field faced in the last decades were also recognised in our work that summarized the 2548 stem cell transplantations, which were performed in Hungary in the period between 1993 and 2015 (22).

2.1.1.3. Improving survival over time

The survival following allo-HSCT has been improving. The Fred Hutchinson Cancer Center, one of the leading transplant hospitals in the world published data confirming better post-transplant survival by time (8). Outcome data for 1993-97 and 2003-07 were compared. Although patients transplanted in the later era were older and had more advanced disease significant decreases in mortality, in severe GvHD, in viral, bacterial and fungal infections and in organ damage were observed. Overall mortality reduced by 41%. Authors put the improved results down to the several changes implemented in their transplantation practice and the consequent reductions in organ toxicity, infection and severe GvHD (8). The US-based National Marrow Donor Program (NMDP) reported outcome data of 15059 unrelated transplants in 2015 comparing transplants conducted between 2000-2004 and 2005-2009 (23). Four groups were distinguished; transplants for malignant disease in patients aged below 18, 18-59, 60 or above and for non-malignant disease at any age. Three-year overall survival (OS) rates were significantly better in all subgroups in 2005-2009: 55% vs. 45%, 42% vs. 35%, 35% vs. 25% and 69% vs. 60%, respectively. The absolute survival improvement was meaningful (7 to 10% in the different categories). Authors emphasised that the significant survival improvement

achieved within a decade likely related to better patient and donor selection and transplantation practice changes including better control of severe GvHD (23).

2.1.2. Indications of allogeneic HSCT

EBMT regularly publish recommendations on the indications of transplantation, of which the most recent one was released in 2015 (3). For indications where transplantation is regarded as standard of care the results of transplantation are superior to other modalities and therefore transplantation should be highly considered. According to the EBMT survey published in 2014 16946 allogeneic haematopoietic stem cell transplants were performed in the EBMT reporting countries in a year (4). The major disease indications consisted of acute myeloid and lymphoid leukaemias (36% AML, 16% ALL), other haematology malignancies (35%) and benign haematological conditions (11%) with only low numbers of other pathologies such as autoimmune disease or solid tumour (4).

2.1.3. Short and long term complications of allogeneic HSCT

Although allogeneic HSCT is considered the only curative option for many conditions, the procedure is associated with significant transplant-related morbidity, mortality and long term health issues (11). Among transplant-specific acute complications acute GvHD is the most important accounting for high mortality (24). Various forms of infections and infectious complications (bacterial, fungal, viral, with special significance cytomegalovirus [CMV] reactivation and Epstein-Barr virus [EBV]-associated post-transplant lymphoproliferative disorder [PTLD]) can develop in patients who are extremely vulnerable secondary to profound immunodeficiency (25). Infections are responsible for significant transplant-related mortality (TRM), especially in the early post-transplant period (9). Other less common, but severe side effects of the procedure include sinusoidal obstruction syndrome (SOS), previously known as veno-occlusive disease (VOD) and transplantation-associated thrombotic microangiopathy (TA-TMA) (11, 26). Among long-term complications chronic GvHD (cGvHD) is of utmost prognostic significance, which can severely diminish transplant survivors` quality of life and result in permanent disability and significant mortality (27). Organ dysfunctions (cardiac, pulmonary, renal, endocrine, musculoskeletal etc.), infertility and secondary cancers are significantly more prevalent among allo-HSCT survivors compared to the

general population, justifying why patients require life-long follow-up preferentially in the long term follow up transplant clinic where their specific needs can be addressed (28, 29).

2.2. GRAFT-VERSUS-HOST DISEASE

Graft-versus-host disease remains the major limitation to allo-HSCT efficacy accounting for significant morbidity and mortality (30). Following relapse or progression of the underlying disease GvHD is the second most common cause of death in transplant recipients with a fatality rate of almost 20% (30).

GvHD is a result of immunological attack on recipient targets delivered by alloreactive T cells (31). GvHD was first described as a syndrome by Billingham more than fifty years ago and the so called Billingham's postulates determined three prerequisites for its development: (1) the presence of donor immunocompetent cells, (2) immunocompromised recipient with incapability of eliminating the donor cells and (3) histocompatibility disparity between the recipient and the donor (32).

2.2.1. Classification of GvHD

GvHD can present in two major forms, the acute and chronic (27). According to the previous algorithms all forms of GvHD, which developed within 100 days post-transplant were classified as acute GvHD, and any forms of alloimmunity affecting patients following day 100 were considered as chronic GvHD (33). The 2005 National Institute of Health Consensus Conference redefined acute and chronic GvHD based on their distinct pathogenetic and clinical features independently of the timing of presentation (34). Subsequently revisions have been published to address areas of controversy (27). Acute GvHD is characterized by inflammatory components, whereas chronic GvHD by autoimmune and fibrotic elements resembling various autoimmune conditions, such as systemic sclerosis or Sjögren's syndrome (31, 34). Acute GvHD has been further sub-classified into classic aGvHD presenting within 100 days after HSCT or donor lymphocyte infusion (DLI) and persistent, recurrent or late-onset aGvHD occurring beyond 100 days (34, 27). Occasionally patients can suffer from overlap cGvHD when the symptoms of acute and chronic GvHD are concurrently present (27).

Based on the current recommendations two subtypes can be distinguished within the acute and chronic GvHD main categories.

1. *Classic acute GvHD*: symptoms of aGvHD present within 100 days of transplant or DLI, without features of cGvHD;
2. *Late acute GvHD*: characteristics of classic aGvHD occurring beyond 100 days post-transplant or DLI;
    - Persistent acute GvHD: aGvHD development within 100 days post-transplant or DLI, but continuation of symptoms after 100 days;
    - Recurrent acute GvHD: recurrence of previously resolved classic aGvHD beyond 100 days after transplantation or DLI;
    - Late-onset acute GvHD: entailing typical signs of aGvHD, but with first onset beyond 100 days;
3. *Classic chronic GvHD*: diagnostic and distinctive features of cGvHD without components of aGvHD manifesting at any time post-transplant;
4. *Overlap chronic GvHD*: one or more aGvHD manifestation in a patient with cGvHD (27, 33).

As this work investigated associations with acute GvHD, chronic GvHD is not discussed in details.

2.2.2. Clinical presentation and diagnosis of acute GvHD

2.2.2.1. Clinical presentation

Acute GvHD typically starts coinciding with or soon after the engraftment of donor cells. However it can develop as early as within two weeks post-transplant prior to engraftment in the form of hyperacute GvHD (24, 35). The three principal target organs of the immunological damage are the skin, the liver and the gastrointestinal tract manifesting as maculopapular skin rash, nausea, vomiting, diarrhoea, abdominal pain, ileus and cholestatic hyperbilirubinaemia (24). Cutaneous GvHD is the earliest and most common presentation of aGvHD (24). The rash, which is typically a maculopapular one, initially develops on the palms, soles and neck, but can start at any site and spread around the entire body (36, 37). Eventually the rash can become generalised, confluent and progress to an extent showing signs of bullous eruption, ulceration and exfoliative dermatitis resembling Stevens-Johnson syndrome (37). Liver aGvHD manifests in the form of liver dysfunction suggestive of a cholestatic picture with high alkaline phosphatase, gamma-

glutamyl-transpeptidase and bilirubin levels (36). The findings reflect on the immunologic damage of the bile canaliculi (38). Patients often suffer from associated symptoms such as pruritus caused by cutaneous and/or liver involvement (36, 37). Gastrointestinal aGvHD typically presents as diarrhoea with or without abdominal pain. It has been increasingly recognised that upper GI GvHD is also a common manifestation of the disease resulting in anorexia, nausea and vomiting (24, 38). Secondary to the epithelial damage and ulceration patients can develop GI bleeding that confers a very dismal prognosis (24, 38). In a report at onset of aGvHD the most commonly affected site was the skin (81%), followed by GI tract (54%) and liver (50%) (39).

The diagnosis of aGvHD is based on clinical grounds in a patient presenting with typical features following allo-HSCT (36). Nonetheless the symptoms are not exclusive to aGvHD, and many conditions post-transplant can induce similar signs or symptoms, therefore the diagnosis is frequently one of exclusion (36). The differential diagnosis for every manifestation of aGvHD is broad comprehending bacterial and viral infections, drug toxicities, side effects of conditioning, SOS, engraftment syndrome. Histological confirmation is supportive of the clinical suspicion, but the treatment should not be withheld until biopsy-proven confirmation bearing in mind that biopsy can even be false negative. Skin biopsy is a routine procedure and in most cases GI biopsy can also be readily obtained, but liver biopsy poses a high risk of complications (24).

2.2.2.2. Diagnostic criteria

The severity of aGvHD can be determined by the extent of organ involvement in the above three target organs. The modified Glucksberg grading system is the most widely applied tool for these purposes (**Tables 1 and 2.**) (40, 41).

Table 1. Staging of aGvHD

Stage	Skin	Liver	GI tract
0	No GvHD rash	Bilirubin <2 mg/dl or <34 μmol/l	No symptoms
I	Maculopapular rash <25% of body surface area (BSA)	Bilirubin 2-3 mg/dl or 34–51 μmol/l	Diarrhoea 500-1000 ml/day
II	Maculopapular rash over 25-50% of BSA	Bilirubin 3.1-6 mg/dl or 52–102 μmol/l	Diarrhoea 1000-1500 ml/day
III	Maculopapular rash >50% of BSA	Bilirubin 6.1-15 mg/dl or 103–256 μmol/l	Diarrhoea > 1500 ml/day
IV	Generalised erythroderma with bullous formation and/or desquamation	Bilirubin >15 mg/dl or >256 μmol/l	Severe abdominal pain with or without ileus.

Taking into account the stage of each organ involvement an overall grade can be established, which has critical prognostic implications. Overall grade I disease is classified as mild, grade II as moderate, grade III as severe and grade IV as life-threatening aGvHD.

Table 2. Grading of aGvHD

Grade	Stage of skin involvement	Stage of liver involvement	Stage of GI tract involvement
0 No aGvHD	0	0	0
I Mild aGvHD	I-II	0	0
II Moderate aGvHD	III	I	I
III Severe aGvHD	-	II-III	II-IV
IV Life-threatening aGvHD	IV	IV	-

2.2.3. Risks factors of acute GvHD

Acute graft-versus-host disease affects 20-80% of patients undergoing allo-HSCT (42). Given the dismal prognosis of severe aGvHD and that there have been no great advances in the clinical management of aGvHD upon its development, attempts have been made to determine factors that could predict the occurrence of aGvHD. As a result of extensive research focusing on the identification of risk factors, with special view to the potentially avoidable ones several clinical, biological and genetic factors have been established (43). Many of these factors, such as patients' age or underlying disease are non-modifiable factors, whereas others including graft source, conditioning and GvHD prophylaxis can potentially be targeted according to patient's individual needs (44).

2.2.3.1. Ethnicity

Populations that encountered less immigration during history and remained isolated for prolonged periods, called island populations, are more likely to have less genetic diversity (45). In the early transplant era authors reported low incidence of aGvHD in Japanese leukaemia patients and suggested that the findings might be explained by genetic homogeneity of the Japanese population (46). Research groups therefore hypothesised that ethnicity could affect the development of allo-HSCT and the reduced diversity of histocompatibility antigens could lead to less GvHD and potentially result in improved survival (45). This speculation was confirmed in a study showing that white Americans, African Americans and Irish cohorts developed more aGvHD and had higher risk for early TRM in comparison with Japanese and Scandinavian cohorts, though the study failed to detect an OS difference (45). The study revealed that the African American population had the most, the Japanese the least diverse HLA representation and the others had an intermediate variability, which is likely a surrogate for the complexity of other genes such as minor histocompatibility genes and cytokine gene polymorphisms that also play a role in graft versus host reactions (45). Authors highlighted that results should be interpreted with caution as not only genetic, but other factors could also contribute to the observed alterations such as dietary or socioeconomic factors and differences in transplant techniques and patient care (45).

2.2.3.2. Disease indication and stage of disease

In a sibling cohort the diagnosis of CML vs. acute leukaemia was associated with more aGvHD and authors speculated that this might have been related to higher tumour necrosis factor alpha (TNFA) serum levels documented in CML, which could facilitate cytokine storm and aGvHD (47). Contrarily, CML was protective for the development of aGvHD in a mixed related and unrelated transplant cohort (48). A third study did not find the diagnosis relevant in the sibling subset, CML was associated with high aGvHD incidence only in the unrelated setting as were all disease types other than ALL (44). In the same report more advanced disease status also interacted with higher aGvHD risk (44).

2.2.3.3. Recipient and donor age

Older patient age has been reported to be correlated with higher risk of aGvHD in several studies, while not all have confirmed an association (47, 49-51). One theory to explain this observation is that tolerance declines during aging secondary to thymic involution and loss of thymic epithelial cells. This may lead to dysregulation of regulatory T cells (Tregs), involved in immunological tolerance, and deficient negative selection of donor originated anti-host T cells (52).

The role of donor age remains a controversial area. In a large cohort of 6978 unrelated donor BMTs facilitated by the NMDP the cumulative incidence of aGvHD grades III-IV was significantly lower with donors aged 18-30 years compared to older age groups (30 vs. 34%, p=0.005) and this translated into better disease-free survival (DFS) and OS (53). Fifteen years later another report was published from the same first author and colleagues at the era of high resolution HLA typing including 6349 transplants in the training and 4690 in the validation cohort. Similarly to the previous observation donor age was significantly associated with aGvHD and OS (54). On the contrary other groups have not found a significant impact of donor age on aGvHD risk (44).

2.2.3.4. Gender mismatch and donor parity

It was recognised two decades ago in an EBMT report that transplant of male recipients with female donors resulted in inferior outcome compared to other sex match constellations (55). Likewise, a later EBMT study confirmed inferior survival and an

increased risk of severe aGvHD in male patients transplanted from female donors (50). Further studies described similar significance of this constellation (48, 44). The findings of other studies suggest that alloimmunisation of female donors during previous pregnancy and parity can contribute to the increased risk. In fact in a large cohort with more than ten thousand transplants the risk was not significantly higher in transplants from nulliparous females compared to males (54, 56).

2.2.3.5. Conditioning intensity and type

The influence of conditioning intensity on aGvHD occurrence has been invariably proven in large number of studies showing reduced risk following reduced intensity (RIC) compared to myeloablative conditioning (MAC), as summarised in a meta-analysis (57). Not only the regimen intensity, but the used modalities also impact on the development of aGvHD and the use of total body irradiation (TBI) has been most consistently linked to aGVHD based on data from large cohorts (48, 47, 44). Comparing the two most commonly applied myeloablative regimens authors found more aGvHD with cyclophosphamide (CY)/TBI conditioning compared with busulfan (BU)/CY (47). Alike the importance of both the intensity and the type of the conditioning was emphasized by a large US study (n=5561) (44). TBI is very potent in the induction of tissue damage, which triggers the release of many inflammatory cytokines that play an important role in aGvHD initiation (58). Gastrointestinal epithelial damage can manifest in the form of diarrhoea and severe mucositis with accompanying pain often results in no or poor oral intake. The relevance of conditioning-related toxicity has been verified by studies, which confirmed that prolonged inability of oral intake and the presence of longer and heavier diarrhoea were very significantly associated with aGvHD occurrence (59, 60).

2.2.3.6. Donor type, stem cell source and dose

Transplantation from matched unrelated donors (MUD) has been clearly and consistently associated with increased risk of aGvHD compared with matched related donor (MRD) transplants (48, 50, 51). Meta-analyses comparing peripheral blood stem cell- (PBSCT) and bone marrow transplantation (BMT) have consistently determined increased cGvHD incidence with PBSCT, but association has not been so clearly documented with view to aGvHD, however a number of reports suggest that the risk of aGvHD might also be higher

(61, 62, 47, 63, 54, 51). Studies investigating the risk of GvHD following umbilical cord blood transplantation agreed that the risk was considerably less with the use of cord blood as opposed to other sources even if most of the umbilical cord blood transplantations are performed with HLA mismatched grafts (62, 64, 65). Studies have showed great inconsistencies concerning the association of GvHD risk and CD34+ stem cell dose, and the correlations have mainly been observed with regard to cGvHD and not aGvHD (66-69). Most studies have found no association with aGvHD, while one found higher, whereas another lower aGvHD risk in association with higher CD34+ stem cell dose (70, 68, 71, 72).

2.2.3.7. CMV serostatus and matching

CMV reactivation is responsible for significant morbidity post-transplant and large studies have revealed the impact of recipient and donor CMV serostatus compatibility on survival (73, 51). In a massive EBMT study assessing data on 49542 transplants CMV serostatus matching significantly influenced survival in the MUD setting corroborating inferior OS in CMV seronegative recipients grafted from a CMV seropositive donor and in CMV seropositive patients transplanted from CMV seronegative donors with MAC (73). Such difference was not seen in the sibling transplant scenario (73). Occasional studies have indicated that CMV seropositivity in the recipient and/or donor could also affect aGvHD manifestation, but in a recent publication of 9469 transplants no independent association with donor/recipient serostatus or CMV disease could have been elucidated (47, 51).

2.2.3.8. GvHD prophylactic agents

The protective role of adding cyclosporine (CSA) to methotrexate (MTX) for aGvHD development was proved decades ago, and consequently calcineurin inhibitors (CSA or tacrolimus [TAC]) plus MTX has become the standard combination for aGvHD prophylaxis at most centres (74, 75). Previous studies looked into whether the calcineurin inhibitor (CNI) choice could impact on the risk of aGvHD and found lower aGvHD incidence with TAC as opposed to CSA combinations (44, 54, 76). The T lymphocyte content of the graft is relevant for GvHD. The protective role of in vivo T cell depletion by anti-thymocyta globulin (ATG) or alemtuzumab has been also affirmed by many

research groups. (64, 77, 48, 78, 79). On the other hand studies found higher relapse risk both with alemtuzumab and ATG resulting in decreased DFS (78). As proven with regards to many concepts in the transplant field, no approach comes without a cost and this clearly implies for in vivo TC depletion, which is associated with delayed haematopoietic and immune reconstitution, increased risk of viral infections and consequent complications, such as EBV-driven PTLD and possibly with higher risk of relapse especially if higher doses are used (80). Considering that graft-versus-host and graft-versus-tumour effects often come alongside, transplant physicians must weigh the risks in the individual patient and target the treatment accordingly.

2.2.3.9. Other clinical factors

Occasional reports have implicated the role of ABO blood group mismatch on aGvHD risk, but this is among the least consistently verified factors (44, 81, 82). In recent analysis involving 11364 patients with acute leukemia registered in the EBMT megafile authors certified that EBV seropositivity of the donor, but not recipient, increased the risk of acute and chronic GvHD, although the increased risk of aGvHD was low, the impact on cGvHD was substantial (83).

2.2.3.10. Genetic factors

2.2.3.10.1. The role of HLA genes

The most influential genetic factor on the outcome of allo-HSCT and on the incidence of aGvHD is major histocompatibility complex (MHC) disparity between the recipient and donor (84). Based on the recipient and donor HLA typing results a well matched donor can be defined as 8/8, 10/10 or 12/12 matched with the recipient, whereby HLA-A -B, -C and HLA-DRB1 loci are taken into account for 8/8 matching, additionally HLA-DQB1 for 10/10 and HLA-DPB1 for 12/12 matching (85, 7). A HLA mismatched donor refers to a donor who has disparity for one or more antigens or alleles of the HLA genes with the recipient (49).

It has been clearly validated by many, large studies that a single mismatch at HLA-A, -B, -C and HLA-DRB1 antigens or alleles increases the risk of GvHD and has a strong negative influence on survival. The relative impact of mismatches at the individual loci

however seem to vary across the studies (86, 49, 87, 7). The clinical relevance of single HLA-DQB1 mismatch remains uncertain (84). The role of HLA-DPB1 as transplantation antigen has recently been elucidated, and studies suggest that some mismatches can be permissive (88, 89). The compounding deleterious effect of multiple mismatches on GvHD presentation and mortality has been demonstrated in several cohorts (86, 49, 90). Equivalently to the MAC transplants, HLA mismatching in the RIC setting has been associated with increased incidence of aGvHD, TRM and lower DFS and OS (91, 92).

2.2.3.10.2. The role of non-HLA genes

Acute GvHD remains a clinical problem even in the well HLA-matched sibling donor setting underlining the significance of non-HLA genes in the alloimmune reaction. To date, hundreds of genetic variants of innate immune receptors, cytokine-, chemokine-mediated pathway genes, apoptosis-related pathway genes, genes involved in drug metabolism etc. have been tested in relation to HSCT outcomes (93-96). Among these genes the most commonly investigated genes were the cytokine genes and findings revealed the association with outcome for many of them, especially in the sibling HSCT setting, where additional non-MHC antigens could contribute to GvHD risk more significantly. Polymorphisms in genes and/or their receptors of TNFA, interleukin (IL)-1, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17, IL-18, IL-23, interferon gamma (IFNG) and transforming growth factor beta 1 (TGFB1) of the recipient and/or donor have been reported in association with the occurrence and severity of acute and/or chronic GvHD, relapse rate and survival (97, 98, 95, 99, 96). **Table 3.** highlights some of the cytokine/chemokine gene polymorphisms, which have been found to be associated with aGvHD development or severity.

Table 3. Gene variants of cytokine and chemokine genes associated with aGvHD development

Gene	Variant	R/D	n	MRD/ MUD	aGvHD occurrence (any, II-IV, III-IV grades)	Ref
CCR9	rs12721497 (926Gu>A)	R	167	MRD	AGu: ↑ (cutaneous)	(100)
IFNG	rs2069705 (1615C>T)	D	1058	MUD	CT or CC: ↓	(101)
IL-2	rs2069762 (330T>Gu)	R	95	MUD	Gu: ↑	(102)
IL-6	rs1800795 (174Gu>C)	R	166	Mixed	GuGu: ↑ in MRD	(103)
IL-6	rs1800795 (174Gu>C)	D	160	MRD	GuGu: ↑	(104)
IL-6	rs1800797 (597Gu>A)	R	166	Mixed	GuGu: ↑ in MRD	(103)
IL-7RA	rs1494555 (1237A>Gu)	D	590	MUD	GuGu: ↑	(105)
IL-7RA	rs1494558 (510C>T)	D	590	MUD	TT: ↑	(105)
IL-10	rs1800871 (819T>C)	R,D	138+102	Both	R, D CC: ↑	(106)
IL-10	rs1800872 (592A>C)	R	570	MRD	AA: ↓	(107)
IL-10	rs1800872 (592A>C)	R,D	138+102	Both	CC: ↑	(106)
IL-10RB	c238A>Gu	D	953	MRD	Gu: ↓	(108)
IL-17	rs2275913 (197Gu>A)	R	510	MUD	A: ↑	(109)
IL-23R	1142Gu>A	D	221+186	Both	Gu: ↓	(110)
TGFB1	rs1800469 (1347C>T)	R	394	Mixed	TT and CT: ↑ (cutaneous)	(95)
TGFB1	rs1800469 (1347C>T)	R,D	138+102	Both	D TT, R T: ↓ in MUD D TT: ↓ in MUD+MRD	(106)
TGFB1	rs1982073 (29T>C)	R,D	427	MUD	Non-wild type R/D pair: ↑	(111)
TNFA	rs1799724 (857C>T)	D	138	MUD	CC: ↑	(112)
TNFA	Rs1800610 (488Gu>A)	R	160	MRD	A: ↑	(104)
TNFB	rs909253 (252A>Gu)	R	138	MUD	Gu: ↑	(112)
TNFII	rs3397 (C/T)	D	394+87	Both	C: ↑	(113)

Column R/D indicates whether the genetic variant of the recipient, the donor or both showed significant association with aGvHD. In column MRD/MUD the term mixed is used for cohorts where analyses were conducted combined for related and unrelated transplants and both if separate analyses were performed for MRD and MUD cohorts (often as discovery and validation cohorts). ↓ indicates less, ↑ more or more severe aGvHD.

Abbreviations for Table 3. A=adenine, aGvHD=acute graft-versus-host disease, C=cytosine, CCR9= C-C motif chemokine receptor 9, D=donor, Gu=guanine, IFNG=interferon gamma, IL=interleukin, IL-2=interleukin-2, IL-6=interleukin-6, IL-7RA= Interleukin-7 receptor subunit alpha, IL-10=interleukin-10, IL-10RB=interleukin-10 receptor subunit beta, IL-17=interleukin-17, IL-23R=interleukin-23 receptor, MRD=matched related donor, MUD=matched unrelated donor, n=number, R=recipient, Ref=reference, T=thymine, TGFB1= transforming growth factor beta 1, TNFA=tumour necrosis factor alpha, TNFB=tumour necrosis factor beta, TNFRII= tumour necrosis factor receptor 2.

2.2.4. The pathomechanism of acute GvHD

The classical pathophysiological model by Ferrara described aGvHD as a three-step process, including tissue damage in the host as first phase induced by pre-transplant preparative regimens (chemo-, and/or radiotherapy) (24). In the second step the activation of recipient and donor originated antigen-presenting cells is the key leading to activation and expansion of donor T-cells that in the third effector phase mediate cytotoxicity against host cells (24). In the first phase the gut epithelium plays an integral role explaining why certain conditioning regimens, such as high dose radiation indicate greater aGvHD risk by compromising integrity of mucosal barriers allowing bacterial endotoxins to translocate into the circulation and trigger the release of pro-inflammatory cytokines (58). Injured tissues respond by upregulated expression of adhesion molecules, co-stimulatory molecules and histocompatibility antigens on host APCs that enhances antigen presentation and induction of GvHD (114-116). This phase is often referred to as “cytokine storm” (31, 117). Beyond the conditioning regimen other factors contribute to the balance between pro-, and anti-inflammatory cytokines and the damage caused, such as patient co-morbidities, the parameters of the graft (source, number of stem cells and T cells) and the applied GvHD prophylactic protocol (24).

The essential role of antigen presenting cells (APC) is well-established giving that the presentation of disparate major and/or minor histocompatibility antigens on APCs leads to the activation of donor T cell receptors (52). Research suggests that however both recipient and donor APCs can induce activation host APCs are significantly more potent (118). Haematopoietic as well as non-haematopoietic APCs can prime T cells (52). Although T cell receptor activation is a crucial step for effector immune responses the initial signal on its own is insufficient and would result in anergy and apoptosis (52). Additional co-stimulatory signalling events are required to induce T cell activation and proliferation (119, 52). One example of co-stimulation is the cluster of differentiation (CD) 40 molecule on the APC and its corresponding ligand the CD40L (cluster of differentiation 40 ligand) on the T cell (52). On the other hand certain receptor-ligand interactions relay inhibitory signals, among which the most studied one is cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and CD80/86 (52). Our knowledge on the cellular subsets interacting in aGvHD has become increasingly complex (31). Although the participation of T helper cell 1 and 17 (Th1 and Th17) cells is still thought to be critical, findings suggest that T helper cell 2 (Th2)-type immune responses are also involved, and tolerogenic pathways are getting better defined (31). The maturation and differentiation of naïve T cells are largely influenced by the local cytokine milieu, the type and dose of the cytokine, the timing of the transplantation and individual responses are dependent on the genetic heterogeneity of the host and the donor (31). Due to the extreme complexity of the cellular interplay involving innate immune cells and many subsets of T and B cells, their detailed description would be beyond the scope of this work (31).

The production and release of pro-inflammatory cytokines, such as TNFA, IL-1, IL-2, IL-6, IFNG remain crucial throughout the three pathophysiological phases of aGvHD with a dual action of direct toxicity to target tissues and the amplification of the donor immune responses against the host cells (120). Cytokines are fundamental for sustained T cell activation (52). The relationship of the partakers that induce and exaggerate aGvHD is self-perpetuating, as cytokines promote T cell activation and expansion, which in turn stimulate further cytokine release (120). Although the Ferrara model is schematic and has its own limitations, the significant contribution of the so called “cytokine storm” to the

development of aGvHD is clearly demonstrated by the model and therefore it still remains valid.

Biomarker studies highly authenticate the role of cytokines in the pathomechanism of aGvHD. In studies high serum levels of TNFA pre-transplant were associated with more severe GvHD post-transplant, and increase in plasma TNF receptor 1 (TNFR1) levels in the first week after MAC allo-HSCT correlated with the incidence and the severity of aGvHD and survival (121, 122). Subsequent research described a four-biomarker panel including IL-2RA, TNFR1, IL-8 and hepatocyte growth factor, and patients who developed aGvHD had significantly higher levels of these markers by a median of 29 days post-transplant, compared with those without aGvHD (123). The role of suppression of tumorigenicity 2 (ST2) as a biomarker has recently been elucidated and led to dynamic research in the field of the newly recognised cytokine axis of ST2/IL-33 (124). Authors found that plasma ST2 levels at day 14 post-transplant were predictive for 6-month non-relapse mortality (NRM) and levels at initiation of therapy for aGvHD showed correlation with resistance to GvHD therapy (124). ST2 is the receptor for IL-33, a member of the IL-1 superfamily that can have both pro-, and anti-inflammatory effects (125). Research proved increased ST2 and IL-33 levels following conditioning and in GvHD with resultant potent activation of T cells (125).

To validate the significance of cytokines in the process of GvHD clinical trials have showed promising results by cytokine pathway-targeted therapies, such as anti-TNF medications (30).

2.2.5. Prevention, treatment and prognosis of acute GvHD

2.2.5.1. Acute GvHD preventative strategies

Before sufficient immunosuppressive measures were implemented the successful performance of allo-HSCT had been jeopardised by extremely high graft failure/rejection and GvHD rates (21). Methotrexate (MTX) has been used for over 60 years and it still remains the component of standard GvHD prophylaxis (126). Calcineurin inhibitors (CNI), cyclosporine (CSA) and tacrolimus (TAC) are the backbone of pharmacological immunosuppression following allo-HSCT at current times (75). The first publication on

the successful administration of CSA with MTX for GvHD prevention in humans comes from 1980, which certified its favourable impact on both aGvHD incidence and survival (127). The protective capacity of CSA was validated in subsequent clinical trials followed by worldwide employment (128). About two decades later TAC, another calcineurin inhibitor entered the clinical stage and it was ratified superior to CSA with regard to aGvHD preventative efficacy, however this potency did not result in improved OS and contradictory one of the studies showed inferior survival with TAC (129, 76, 130). Mycophenolate mofetil (MMF) has been a frequently used alternative agent in preventative combinations (126). The immunosuppressive potential of sirolimus (SIR) in aGvHD prevention was first reported in 2003. Authors added SIR to the standard TAC/MTX combination and observed an unusually low aGvHD grades II-IV rate of 26% (131). In a randomised controlled trial (RCT) TAC/SIR combination led to less aGvHD in comparison with TAC/MTX, but no improvement was found in OS (132). Several other studies affirmed similar associations, but also brought the transplant physicians' attention to the different side effect profile and especially to the higher risk of TA-TMA and SOS (133-135). Overall since the introduction of CNI-based prophylaxis in the 1980s no great progress in GvHD prevention has been made despite the high number of agents tested (75).

2.2.5.2. Treatment and prognosis of acute GvHD

The clinical severity of aGvHD significantly impacts on outcome. The survival of patients with grade I or II aGvHD is comparable to those without aGvHD, but the overall survival in grade III and IV disease is slim, 25% and 5% at 5 years, respectively (136).

The management of grade I aGvHD includes optimisation of GvHD preventative agents (CNI) and topical corticosteroids or CNI without the need for systemic immunosuppressive drugs (36). Grades II-IV aGvHD should be treated with systemic corticosteroids as first line therapy (36). Slightly less than half of the patients achieve remission with first line treatment (137). Many other agents have been tested as first line options, but none has proved to be superior to corticosteroids (137). The response to steroids directly correlates with survival and the prognosis of steroid-refractory aGvHD is poor with a mortality rate around 70% (137, 138). The list of second and third line

treatment options is extensive. The British Committee for Standards in Haematology guideline recommends the use of the following modalities for second line treatment: extracorporeal photopheresis, anti-TNF antibodies, mTOR inhibitors, MMF, IL-2 receptor antibodies and mesenchymal stem cells, alemtuzumab, pentostatin and MTX for third line management (36). Recently there have been very promising results with the administration of JAK inhibitors for both acute and chronic manifestations of GvHD (139). Numerous other cellular and pharmaceutical agents have been tested with variable response rates (31, 30).

2.3. TRANSPLANTATION-ASSOCIATED THROMBOTIC MICROANGIOPATHY

Haematopoietic stem cell transplant-associated thrombotic microangiopathy (TA-TMA) is a well-documented complication of HSCT with high mortality rate and the risk of long-term health implications in survivals (140). Although TA-TMA has been described for more than three decades, the exact pathomechanism remains unclear (141). Endothelial dysfunction/injury is the key element that induces platelet activation and formation of platelet-rich thrombi in the microcirculation leading to the classical picture of thrombocytopenia secondary to platelet consumption and microangiopathic haemolytic anaemia caused by mechanical damage to red blood cells (142). The small vessel injury often leads to subsequent tissue/organ damage with the kidneys being the predilection site, but in most severe cases the condition manifests as a multi-organ disease with extremely dismal outcome (140, 143).

2.3.1. Classification of TMAs

The term thrombotic microangiopathy (TMA) defines conditions associated with microangiopathic haemolytic anaemia and thrombocytopenia (MAHAT) (144). TMAs are all characterised by a common end point of microthrombotic endothelial damage, but via different mechanisms and triggers (145). Haemolysis is secondary to mechanical damage caused by blood clots in the small vessels (145). Laboratory tests including fragmented red blood cells (schistocytes), increased lactate dehydrogenase (LDH), low haptoglobin, reticulocytosis, negative Coombs test and thrombocytopenia support the diagnosis of MAHAT (144). Microthrombi formation can lead to organ dysfunction in virtually any organ (144).

The latest consensus guideline divides TMAs into three main categories, thrombotic thrombocytopenic purpura (TTP), haemolytic uraemic syndrome (HUS) and other conditions associated with TMA presenting with MAHAT (144). TA-TMA belongs to the latter category and is appreciated as a distinct disorder developing in the setting of haematopoietic stem cell transplantation (145, 144). Despite the etiologic differences, TA-TMA shares clinical and histological similarities with other forms of TMA and the

clinical presentation of various conditions associated with TMA might be indistinguishable (145).

The classical form of TTP, the immune mediated TTP is attributed to an autoimmune deficiency of von Willebrand factor-cleaving protease, known as ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13), which leads to coagulation activation and intravascular thrombus formation (146). Secondary, immune-mediated TTP can be precipitated by heterogeneous triggers such as infections, pregnancy and drugs and can accompany autoimmune or malignant conditions (144). As genetic predisposition, a strong association of TTP with HLA-DRB1*11 has been described in recent years by independent groups, and some other class II HLA genes and haplotypes have also been found accumulated (147-150). The classical pentad of fever, microangiopathic haemolytic anemia, thrombocytopenia, neurologic signs and symptoms and renal failure proposed for the diagnosis of TTP in 1966 is rarely present, and MAHAT are considered sufficient for the presumptive diagnosis of TTP (146). Following the recognition of the pathomechanism, the implementation of therapeutic plasma exchange (TPE) that combines two benefits, the removal of antibodies and the replacement of the deficient protease, has changed the landscape of TTP and reduced its mortality from 85-95% to 10-20% (146).

Renal injury is a hallmark of HUS, which can present in two major forms, the infection-associated and the complement-mediated HUS (CM-HUS) (144). The former typically occurs following Escherichia coli infection in children, but several other organisms have been implicated in its development (145). CM-HUS was formerly known as atypical HUS, but the most recent consensus guideline published in 2017 on the standardization of terminology of TMAs recommends the use of the term CM-HUS (144). CM-HUS results from complement dysregulation of the alternative complement pathway and the clinical manifestation can be triggered by infections, vaccinations and pregnancy (144). Various mutations have been identified in genes of complement factors and associated proteins present in more than half of the cases (149). In cases especially with homozygous deletions of genes autoantibodies, such as anti-complement factor H (CFH) can develop (144). The identification of the pathomechanism has prompted the implementation of antibody depleting and complement blocking agents with great success (145).

2.3.2. Clinical presentation and diagnosis of TA-TMA

2.3.2.1. Clinical presentation of TA-TMA

TA-TMA is one of the most frequent and severe post-transplant complications, although reported incidence shows great variation (0-75%). This is partly due to the lack of uniform diagnostic criteria (140, 151, 152). The complication is likely underdiagnosed and a recent prospective study reported an incidence of 39%, a rate above the documented in most retrospective studies (153). The diagnosis of TA-TMA can be challenging even for experienced transplant physicians given the diverse and non-specific signs and symptoms and the fact that patients often suffer from several complications at the same time with overlapping symptoms. The above explain the frequent late or post-mortem diagnoses (143). TA-TMA can be mistaken for other transplant-related complications associated with endothelial cell damage such as drug toxicity, engraftment syndrome, GvHD, infections, SOS, diffuse alveolar haemorrhage or capillary leak syndrome (140, 154). Over 90% of cases occur within 100 days of transplant and the median time to onset is 30-45 days post-transplant (140). TA-TMA clinically presents as microangiopathic haemolytic anaemia and thrombocytopenia with or without secondary organ damage. As a consequence of haemolysis fragmented red cells (schistocytes or fragmentocytes) can be detected on the peripheral blood film. Thrombocytopenia is secondary to microthrombi formation, which latter is the major cause of organ dysfunction. Kidneys are the predominant site of organ involvement, but any organ can be affected, including the gastrointestinal (GI) tract, central nervous system and pulmonary vasculature. (143). Signs of renal damage, such as proteinuria and hypertension should raise suspicion and can aid earlier diagnosis (143). GI involvement can manifest in diarrhoea, severe abdominal pain, bleeding or ileus (154). Central nervous system damage can cause headache, confusion, hallucinations or seizures (154). Polyserositis can present with resistant pleural, pericardial effusion or ascites (154). The symptoms/signs of TA-TMA are very common in the post-transplant setting for many other reasons, which can be misleading. Tissue diagnosis, which could be confirmatory is not amenable in most cases due to high risk of complications and frailty of the patients (143). Due to limited feasibility of pathological diagnosis the diagnosis of TA-TMA largely relies on clinical grounds and requires a high index of suspicion (154, 143).

2.3.2.2. Diagnostic criteria of TA-TMA

Reflecting the diagnostic challenges and uncertainties in 2004 George et al. found 28 different sets of diagnostic criteria in 35 articles involving 5423 allo-HSCT patients (26). Following the above review several societies have proposed diagnostic tools in order to standardise diagnosis and reporting. The three most widely accepted criteria are listed in **Table 4**. The Blood and Marrow Transplant Clinical Trials Network (BMT CTN) Toxicity Committee recommendations were published in 2005 (151). The diagnostic set incorporated four specifications to be present for diagnosis: 1.) red blood cell fragmentation and ≥ two schistocytes per high-power field (HPF) on peripheral smear; 2.) concurrent increased serum lactate dehydrogenase (LDH) above institutional baseline; 3.) concurrent renal (defined as doubling of serum creatinine from baseline or 50% decrease in creatinine clearance from baseline) and/or neurological dysfunction without other explanations; 4.) negative direct and indirect Coombs test results. The International Working Group consensus criteria were proposed in 2007 by Ruutu et al. and recommended the diagnosis of TA-TMA if all the following criteria were fulfilled: 1.) > 4% schistocytes in blood; 2.) de novo, prolonged or progressive thrombocytopenia (< $50x10^9$/l or 50% or greater reduction from previous counts); 3.) sudden and persistent increase in LDH; 4.) decrease in haemoglobin concentration or increased transfusion requirement; 5.) decrease in serum haptoglobin (155). The third diagnostic set evolved following the exploration of limitations of the two, above tools (156). In a validation study Cho et al found that many cases would have been missed with the application of the above two criteria. In their cohort the cumulative incidence of TA-TMA based on their institutional criteria was 12.7%, on BMT CNT definition 6.1% and on International Working Group recommendation only 2.5%. The group suggested that patients should be diagnosed based on the following criteria: 1.) Normal coagulation assays; 2.) ≥ two schistocytes per HPF; 3.) Increased LDH; 4.) Negative Coombs test; 5.) de novo, prolonged or progressive thrombocytopenia (< $50x10^9$/l or 50% or greater reduction from previous counts); 6.) Decrease in haemoglobin concentration; 7.) Decrease in serum haptoglobin. The group proposed that in the overall TMA category patients with and without organ dysfunction should equally be considered and therefore they distinguished the so called “probable TMA” category for patients with no organ damage as per BMT

CTN TMA criteria. Since its publication this recommendation has become the most widely accepted and cited in papers reporting on TA-TMA.

Table 4. Proposed diagnostic criteria of TA-TMA

	BMT Clinical Trials Network Toxicity Committee (2005)	**International Working Group (2007)**	**Cho et al. (2010)**
Schistocytes	≥ 2 per HPF on peripheral blood smear	> 4% in peripheral blood	≥ 2 per HPF in peripheral blood
LDH	Increased	Sudden and persistent increase	Increased
Red Cells		Decreased haemoglobin or increased red cell transfusion requirement	Decreased haemoglobin
Platelets		Thrombocytopenia	Thrombocytopenia
Organ dysfunction	Renal and/or neurological dysfunction without other explanations		
Coombs Test	Negative direct and indirect		Negative
Haptoglobin		Decreased	Decreased
Other			No coagulopathy

Abbreviations for Table 4. BMT=bone marrow transplant, HPF=high power field, LDH=lactate dehydrogenase.

Jodele et al. have recently proposed a refined diagnostic set including hypertension, proteinuria and activated terminal complement complex (sC5b-9) serum level on top of the already included parameters of LDH, schistocytes, thrombocytopenia and anaemia

(153). Transplant recipients with five of these criteria were very likely to develop multi-organ involvement. The survival of patients who exhibited proteinuria (> 30 mg/dl) and had evidence of terminal complement activation (elevated sC5b-9) at the time of diagnosis was very poor (< 20% at 1 year). Several studies have confirmed that proteinuria is a more informative marker of renal injury compared to rise in serum creatinine (157). However the measurement of complement factors has not been routinely available yet in the clinical setting and therefore the adoption of these criteria for diagnosis is awaited.

2.3.3. Risk factors of TA-TMA

TA-TMA is a multifactorial disorder secondary to a variety of insults. The role of several predisposing risk factors has been addressed, which rarely act in isolation and in most cases a combination of triggers contribute to the clinical picture.

2.3.3.1. Patient-, and disease-related risk factors

Published, non-modifiable patient-related risk factors have been African American race, female gender and older age (142, 158, 159, 152, 160). A recent study has suggested that the different genetic makeup of complement genes might be one of the explanations for the racial disparities (161). Occasional reports implicated higher risk in the presence of lymphoid malignancies and in transplantation at advanced stage disease (142, 162, 159).

2.3.3.2. Transplant-related risk factors

Many studies established an association with unrelated donor type compared to matched related donors (MRD) (159, 152, 160). Numerous procedural characteristics have been found associated with the development of TA-TMA. Studies documented increased risk with myeloablative (MAC) compared to reduced intensity conditioning (RIC) regimens. Both total body irradiation (TBI) and high-dose busulfan (BU) use in the conditioning have been linked (163-167, 160). Among agents used in RIC regimens, fludarabine (FLU) has been the most consistently reported risk factor (142). The increased incidence of TA-TMA with fludarabine-containing regimens is likely related to fludarabine-induced enhanced allogenicity of endothelial cells and direct endothelial toxicity from FLU (168).

The role of GvHD-prophylactic agents have been very extensively studied. Calcineurin inhibitors (CNI, such as cyclosporine [CSA], tacrolimus [TAC]) are the most commonly applied prophylactic agents for the prevention of aGvHD. Many groups have confirmed an association between CNI use and TMA, both in allo-HSCT and solid organ transplantation (145, 152). Mammalian target of rapamycin (mTOR) inhibitors (such as sirolimus, SIR) have also been identified as risk factors for TA-TMA. Studies have suggested higher incidence with the combined use of CNI and mTOR inhibitors, especially when busulfan was part of the conditioning regimen (169-171). Despite the higher incidence and earlier presentation of TA-TMA following the addition of SIR to CNI the prognosis of sirolimus-induced TA-TMA seems to be more favourable (172). Some reports have described increased risk with higher TAC and SIR serum levels emphasizing the importance of optimization of trough levels (162, 166). Sporadic studies have reported higher risk in HLA-, and ABO-mismatched transplants (156, 173). Prior HSCT has also been reported in few studies (162).

2.3.3.3. Complications of transplantation

The association of aGvHD with TA-TMA has been so strong that some authors even suggested that TA-TMA could be a form of GvHD, which however is not the case (143). The conditions can develop independently and the resolution of GvHD does not automatically result in improvement of TA-TMA and on the opposite continuation of immunosuppression might worsen its course. Cho et al. affirmed in patients with aGvHD that concomitant TA-TMA resulted in worse survival verifying the existence of the two conditions as distinct entities (174). In a study evaluating 314 autopsies of patients who died after first allo-HSCT the odds for renal TA-TMA development were almost four times higher among patients who suffered from grades II-IV aGvHD (163). In another smaller autopsy study all six patients whose autopsy was confirmatory of TA-TMA had prior grades III-IV GvHD (175). Many clinical observations have also endorsed the close link with GvHD (156, 162, 166, 152, 160). Sporadic observations have found an association with SOS, as well (176). Infectious complications are very common in allo-HSCT and these complications often coincide with TA-TMA. Several microorganisms have been associated with TA-TMA, including cytomegalovirus (CMV), adenovirus, human herpes virus-6, parvovirus B19, BK virus and Aspergillus species (143).

2.3.3.4. Genetic factors

At the time of our study to our knowledge no known associations between TA-TMA and genetic factors were revealed. In 2016 Jodele et al. published the most extensive genetic study to date in the field that investigated 17 candidate genes involved in complement activation among 77 patients, of whom 34 developed TA-TMA (161). Gene variants were more common in patients with TA-TMA and 65% of patients with TA-TMA carried genetic variants in at least one complement gene as opposed to only 9% of those without TA-TMA. Authors highlighted that although the genetic variant can put patients into higher risk for TA-TMA, environmental stressors are required for the development of TA-TMA. In patients the relative contribution of genetic susceptibility and additional factors may differ and a strong acquired factor might be sufficient to induce TA-TMA in a genetically less susceptible person and inversely in a genetically prone person mild environmental stressors might result in disease manifestation. Interestingly variants in three or more genes were only found in nonwhites, which was associated with high transplant-related mortality. Authors speculated that complement variants protective against Neisseria meningitidis, which is endemic in certain parts of Africa could have offered survival benefit for the patients' ancestors, but such genetic constellation might be disadvantageous in the HSCT setting.

2.3.4. Pathomechanism of TA-TMA

Although the recent scientific progress in the understanding of other forms of TMA has broadened our knowledge in TA-TMA, its pathomechanism is less well elucidated. As opposed to TTP, TA-TMA is not associated with a severely decreased ADAMTS13 activity (142, 141). The exact initiating events and pathogenetic routes remain poorly identified, but microvascular endothelial cell dysfunction and damage seem to be central to the development of TA-TMA (177). Markers of endothelial activation/injury have been found to be elevated in TA-TMA, including von Willebrand's factor, plasminogen activator inhibitor-1 and thrombomodulin (177). The severe endothelial damage is multifactorial, precipitated by many transplant-associated factors such as conditioning regimens, prophylactic immunosuppressive agents, infections or GvHD and possibly inherited tissue susceptibility (157, 141). CNI, infections or GvHD induce direct damage

to endothelial cells, but can trigger further injury indirectly via release of proinflammatory cytokines and coagulation activation (143). The mechanism of activation/damage differs in case of various triggers. CNI induce damage by reducing the production of prostacyclin, nitric oxide and activated protein C and increasing thromboxane A2 (178). Endothelial cells treated with CNI have been shown to release endothelial microparticles that activate the alternative complement pathway (178). Sirolimus may increase TA-TMA risk by delaying repair of damaged endothelium induced by various triggers including CNIs and by decreasing local vascular endothelial growth factor (VEGF) production (179, 143)

In an autopsy study of 314 post-transplant cases renal TA-TMA was significantly associated with grades II-IV aGvHD (163). Authors suggested several explanations for the strong link between aGvHD and TMA. Endothelial injury might be related to circulating pro-inflammatory cytokines released during GvHD responses (163). Endothelial cells are thought to be direct targets of donor T cells involved in GvHD reactions (163). Markers of endothelial injury and coagulation activation are increased in patients with aGvHD suggesting another link between the conditions (163). The fourth hypothesis to support the association with GvHD is related to VEGF, which has known protective effects on the endothelium. Severe forms of aGvHD have been associated with lower levels of VEGF, which makes the endothelium more susceptible to injury (163). Another pathomechanistic route involving VEGF activity is related to adenovirus that expresses a fms-like tyrosine kinase that binds VEGF. By inactivation/inhibition of VEGF the virus can contribute to TMA development (180).

Most recently evidence has been accumulating to support the role of complement dysregulation in the pathogenesis of TA-TMA similarly to HUS. Complement system analysis in six paediatric patients (three following autologous, three following allogeneic HSCT) with TA-TMA showed that five of them had heterozygous complement factor H related 3 and 1 (CFHR3-CFHR1) gene deletions and all three allogeneic transplant recipients had detectable CFH autoantibodies (181). Authors postulated that autoantibody production was likely a response to recipient/donor genotype differences as none of the autologous HSCT recipients developed autoantibodies. The role of complement system in the pathogenesis has been supported by the above mentioned genetic study by Jodele

et al., as well (161). Seventeen candidate genes known to be involved in complement activation were investigated. Gene variants were more frequent in patients with TA-TMA (65% of patients carried genetic variants in at least one complement gene, whereas only 9% without TA-TMA). Authors argued that the genetic variants might not have biological significance during the course of life, except under high level of stress, such as allo-HSCT. Transplant recipients carrying certain variants might develop TA-TMA via complement-mediated endothelial cell injury (157).

2.3.5. Treatment and prognosis of TA-TMA

2.3.5.1. Treatment of TA-TMA

The treatment of TA-TMA is challenging due to the co-existing other pathologies such as severe aGvHD or infections. Current treatment strategies include aggressive treatment of triggers and cessation of causative agents, therapeutic plasma exchange (TPE), rituximab, defibrotide, eculizumab, recombinant thrombomodulin and some other experimental agents (140, 143). On suspicion of TA-TMA dose reduction or discontinuation of CNI and mTOR inhibitors and the use of alternative agents is recommended (143). Response rates up to 63% have been documented with withdrawal of suspected drugs, which is better than responses to TPE or most pharmacological strategies (182). TPE significantly decreased the mortality of TTP from about 85-95% to 10-20% (146). Unfortunately response rates in TA-TMA have been less promising. Ho et al. reviewed 11 studies using TPE and the response rates varied from 0 to 80% (median 36%), but the associated mortality remained high (80%, range 44-100%). In a cohort overall response rate to TPE was 27%, but of note none of the patients with active aGvHD showed any response to the modality suggesting that in cases with concomitant aGvHD TPE might not be efficacious at all (183). Although a subset of patients could possibly benefit from TPE, in general TPE is not recommended for the treatment of TA-TMA as it failed to alter the aggressive disease course and fatality rate (143). There have been case reports and small case series on the successful application of rituximab and out of 15 patients in literature 12 achieved response (184). This approach could be most beneficial in patients with detectable autoantibodies (140). Defibrotide has been proven to protect the endothelium and via modification of coagulation factors and platelet aggregation is has antithrombotic and anti-ischaemic properties (140). In a publication from 2017 data

on 39 patients treated with defibrotide for TA-TMA was promising. Resolution of TA-TMA happened in 77% of cases (185). Currently emerging and most encouraging treatment modality has been the complement C5 blocking agent eculizumab (157). In the first paediatric case report from 2014 four of six patients treated with eculizumab achieved a complete remission (186). The same group in 2016 reported data on 30 patients treated with eculizumab and confirmed that eculizumab-treated patients had significantly better survival than the historical control (62 vs. 9% at 1 year, p=0.0007) (157). In a French study 12 patients were given eculizumab for TA-TMA with neurological and/or renal involvement of whom 58% were refractory to previous TPE. Half of the individuals achieved haematological response and OS was 33% with a median follow-up of 14 months. Active aGvHD at diagnosis was an unfavourable factor on survival (187). Other agents such as danaparoid, recombinant thrombomodulin, statins, bosentan have been used sporadically (188, 184, 143, 189). As outlined above, current treatment options for TA-TMA are suboptimal. Prospective trials and further research are warranted to reverse the unfavourable prognosis of the condition.

2.3.5.2. Prognosis of TA-TMA

TA-TMA has an extremely poor prognosis. In the review by George et al. the reported mortality rates varied from 0 to 100%, with a median of 75% (26). 82% of deceased patients died early, within 3 months of diagnosis among those with reported time of death data (26). In the validation study by Cho et al. 76% of patients with TA-TMA died and the survival was better in patients with probable TA-TMA (who had no organ dysfunction) (156). In the only prospective, paediatric cohort children with TA-TMA had significantly higher non-relapse mortality (NRM) (44 vs. 8%) at 1 year post-transplant in comparison with children without TA-TMA (153). Patients who survived TA-TMA were more likely to suffer from long-term morbidity, especially chronic kidney disease and cardiovascular complications contributing to increased late mortality (143). Many studies have proved poor renal prognosis following TA-TMA (143, 178). In a study the risk of developing chronic kidney disease was 4.3 times higher for patients with TA-TMA and patients with TA-TMA were 9.0 times more likely to have hypertension than patients without TMA (190).

2.4. JAK/STAT PATHWAY AND THE JAK2 46/1 HAPLOTYPE

2.4.1. The JAK/STAT signalling pathway

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway has a critical role in cytokine-mediated immune responses (191). JAK2 is a ubiquitously expressed tyrosine kinase member of the JAK/STAT family, one of the four Janus kinases in the system: JAK1, JAK2, JAK3 and tyrosine kinase 2 (191). The pathway is activated via binding of multiple cytokines controlling cell growth, metabolism, haematopoiesis, inflammation and immunoregulation (192). The dysregulation of the JAK/STAT signalling pathway has been implicated in the development of haematological and solid tumours, through abnormal regulation of cell differentiation, proliferation and apoptosis (193, 194). The link between the pathway and autoimmune conditions has long been discovered, and recently increasing evidence has accumulated to support the critical role of the network in the pathogenesis of aGvHD (31, 192). Inflammatory bowel diseases (IBD) have been among the first inflammatory conditions associated with the pathway that share common features with GvHD (195, 196). In Crohn's disease pro-inflammatory Th1 and Th17 cytokines are upregulated, including IL-23, one of the key activators of Th17 cells (196). Several genes encoding proteins involved in IL-23 signalling and Th17 cell differentiation have been identified as susceptibility genes to Crohn's disease and less so to ulcerative colitis (196). The JAK/STAT pathway, acting as a principal member of the network involved in IL-23 signalling has a major role in inflammatory responses in the gastrointestinal mucosa, and the pathway regulates the balance between pro-, and anti-inflammatory cells (Th1, Th2, Th17, Treg, myeloid cells), critical for intestinal immunity (196). Alike, JAK2 conveys signalling of T-cells in response to a variety of cytokines, such as IFNG, IL-6, IL-12, IL-23, which initiate, maintain and amplify Th1 and Th17 differentiation and expansion, resulting in alloreactivity and organ damage in GvHD (197). As such, JAK2 acts as a gatekeeper of alloimmunity (197). A recent study by Betts et al. proved that STAT3 activation (downstream to JAK) and resultant Th17 accumulation within the GvHD target organ tissues were increased in aGvHD, and showed association with the onset, severity and treatment outcome of aGvHD (195). IFNG receptor (IFNR) signalling is also mediated via JAK1 and JAK2, which likewise has been proved to be upregulated in

activated T cells present in tissues affected by aGvHD (198, 191). IFNR-/- allogeneic donor T cells induce less GvHD, similarly to the effect of pharmacological blockade of JAK1 and JAK2 (198). Experimental and clinical studies suggest that the JAK/STAT pathway may be a potential therapeutic target for the prevention and treatment of aGvHD without undermining the GvL effect (197, 199-201, 139). In the first multicentre human study ruxolitinib, a JAK1/2 inhibitor achieved an impressive 81.5% overall response rate for steroid-refractory aGvHD (139). The discovery that the JAK/STAT pathway is commonly dysregulated in GvHD and the availability of drugs that modulate the activity of the pathway will likely significantly shape the anti-GvHD armoury of the future.

2.4.2. JAK2 46/1 haplotype

The JAK2 gene is part of a 280 kilobase-long linkage disequilibrium block encoded on chromosome 9 that contains two other genes, insulin-like 4 and 6 (INSL4 and ILSL6), which are not expressed in haematopoietic cells (202). Combinations of several SNPs in this region infer nine main distinct haplotypes, of which two haplotypes are nearly completely identical (numbers 46 and 1; designated as 46/1 haplotype) (202). The 46/1 haplotype is present in about 45% of the general population in either hetero- or homozygous forms and studies confirm that this germline variant significantly influences the risk of acquiring somatic mutations (203). In 2009 three independent research groups simultaneously published that the 46/1 haplotype conferred susceptibility to myeloproliferative neoplasms (MPN) (202, 204, 205). The studies elucidated linkage of various SNPs within the haplotype. Linkage disequilibrium refers to an association of two or more genetic markers, where their frequencies are higher than it could be expected just by chance alone, therefore a non-random linkage of antigens or alleles is suspected (206). Olcaydu et al. reported four SNPs, the rs3780367, rs10974944, rs12343867 and rs1159782 referred to as the GGCC haplotype, and of these the last three SNPs showed a complete linkage disequilibrium with each other and the rs3780367 SNP associated with the others in 96-99% of the alleles (205). Jones et al. reported the investigation of 14 SNPs in the JAK2 gene region, marking rs12343867 and rs12340895 as tagging haplotype SNPs (202).

The initial studies clearly demonstrated a strong link between the haplotype and the risk of JAK2 V617F-positive MPN, with predilection of the mutation on the 46/1 allele in most cases (202, 204, 205). Subsequently, the JAK2 46/1 haplotype has also been reported to be associated with increased risk of JAK2 V617F-negative, JAK2 exon 12 and MPL (myeloproliferative leukaemia virus oncogene) mutated MPN, which latter gene is not located on the same chromosome (207-210). Our research group previously identified that carriership for 46/1 haplotype was associated with normal karyotype acute myeloid leukaemia (AML), and other groups documented that JAK2 polymorphism affected treatment outcomes in AML (211-214). Aberrant function of Janus kinases is often found in AML (215, 216). Most recently the haplotype has been showed to be associated with clonal haematopoiesis, which is present in two out of 1000 individuals in the general population (217). Genome-wide association studies, supported by confirmatory investigations reported an association of JAK2 46/1 haplotype with IBD susceptibility and disease behaviour (218, 219). Inflammation-associated carcinogenesis is a well-documented complication in IBD, but beyond the context of IBD, a causal link between chronic inflammation and cancer has become generally accepted (220, 221). Chronic inflammation has recently been proposed as a potential trigger in myeloid cancers, such as AML and MDS (222). In MPNs the inflammation has been suggested both as an initiator and a driver of clonal evolution, accelerated atherosclerosis and second malignancy (223). Sustained inflammation, accompanied by ongoing myeloproliferation might create and maintain a high-risk microenvironment for development of mutations in the myeloid lineage secondary to the perpetual oxidative stress and DNA damage, induced by the inflammation (224). Besides tumour initiation the chronic inflammatory condition gives rise to tumour progression via epigenetic changes and genomic instability (224). Signalling pathways active both in inflammation and cancer including the JAK/STAT pathway are thought to be key links between the two conditions (223).

Although the exact pathomechanism how the germline JAK2 variation predisposes to the acquisition of MPN or AML is yet to be explored, two alternative hypotheses have been suggested for explanation (225). The ”hypermutability hypothesis” advocating for genomic instability at the JAK2 locus can be corroborated by data that in MPN JAK2 V617F mutation preferentially arises on the 46/1 haplotype (204, 205). The ”fertile ground hypothesis” suggests that cells carrying the 46/1 haplotype might have alterations

in JAK2 function compared to cells with the major allele and these cells might acquire selective advantages in cell growth and proliferation upon mutagenesis, resulting in clonal myeloproliferation (202). However, both hypotheses are confronted with unanswered queries (225). Considering the association of the 46/1 haplotype with inflammatory conditions and cancer it is hypothesised that the haplotype may be associated with a functional variant of JAK2 (223, 203, 202).

The mustered evidence on the link of the JAK/STAT pathway with inflammatory conditions and GvHD and that the JAK2 46/1 haplotype predisposes to IBD that has common features with GvHD prompted our investigations for JAK2 46/1 haplotype testing in the context of allo-HSCT.

3. OBJECTIVES

1) To establish a database including detailed patient, donor and transplantation characteristics of all allogeneic haematopoietic stem cell transplantations performed in adult patients for haematological malignancies in Hungary in the period between 2007 and 2013.

2) To test the hypothesis whether carriership for HLA-DRB1*11 correlates with transplantation-associated thrombotic microangiopathy (TA-TMA). This study was initiated by recent publications showing a strong association between HLA-DRB1*11 and thrombotic thrombocytopenic purpura (TTP), another form of thrombotic microangiopathy. Our objectives were to gather detailed clinical information on TA-TMA development, to elucidate its risk factors - including the presumed association with HLA-DRB1*11 - and to investigate its prognostic implications.

3) To assess the association of recipient and donor JAK2 46/1 haplotypes with transplantation outcome; acute graft-versus-host disease (aGvHD), relapse and survival among acute myeloid leukaemia patients transplanted in complete remission. This study was prompted by previous publications revealing the role of the haplotype in inflammation and cancer and the similar pathological attributes of aGvHD and certain autoimmune conditions including inflammatory bowel disease (IBD), in which the JAK/STAT cytokine pathway has principle roles.

4. METHODS

4.1. Patients

The entire allo-HSCT cohort consisted of 425 consecutive adult patients who underwent first allogeneic haematopoietic stem cell transplantation between January 2007 and December 2013 for a haematological malignancy at the Department of Haematology and Stem Cell Transplantation, St Istvan and St Laszlo Hospital, Hungary. The centre was the only transplant centre providing allogeneic HSCT for adult patients in Hungary at the time of the study, therefore all adult patients treated with the modality for a malignancy have been included in the study. Data were collected and analysed retrospectively. There was a slight male predominance (239/425, 56.2%) in the cohort reflecting on the gender distribution difference of some haematological malignancies. The indications of HSCT were the following in order of their frequency; acute myeloid leukaemia (n=157), acute lymphoid leukaemia (n=75), non-Hodgkin lymphoma (n=42), myelodysplastic syndrome (n=35), chronic lymphocytic leukaemia (n=26), multiple myeloma (n=25), chronic myeloid leukaemia (n=24), myeloproliferative neoplasm (n=23) and Hodgkin lymphoma (n=18). Related and unrelated donor sources were close to equal (217/425, 51.1% related and 208/425, 48.9% unrelated). The majority, almost two thirds of the patients, were treated with myeloablative conditioning pre-transplant (267/425, 62.8%). MAC regimens consisted of TBI 12 Gray (Gy) plus cyclophosphamide (CY) 120 mg/kg (n=157); TBI 12 Gy plus etoposide (VP16) 60 mg/kg (n=31); TBI 12 Gy plus melphalan (MEL) 110-140 mg/m^2 (n=20) and busulfan (BU) 16 mg/kg plus CY 120 mg/kg (n=59). The most commonly given RIC regimen was a combination of non-myeloablative dose BU plus fludarabine (FLU) with or without in vivo T cell depletion (variable doses, n=90), the second most commonly applied regimen was the combination of FLU, MEL and ATG or Alemtuzumab (n=28), other regimens were used in 40 patients. GvHD prophylactic regimens were either cyclosporine (CSA, 24%) or tacrolimus (TAC)-based (76%): CSA (n=16), CSA/MTX (n=26), CSA/MMF (n=58), TAC/SIR (n=244), TAC/MMF (n=56) and TAC/MTX (n=25). Conditioning and post-transplantation immunosuppressive regimens for GvHD prevention were employed dependent on disease indication, donor availability, patient's comorbidities, organ functions, risk of relapse and other variables. The serum levels of CSA, TAC and SIR were monitored closely to achieve and maintain

the target trough levels. Dose levels were targeted according to disease and transplant characteristics and the type of combination used. Dose adjustments and modifications of regimens were initiated in the event of toxicities, engraftment failure, GvHD, mixed chimerism or relapse.

Acute GvHD was defined and graded according to consensus criteria (modified Glucksberg, as detailed in **Table 1.** and **2.**) (40, 41).

Standard karyotyping with at least 20 analysed metaphases was part of the routine diagnostic workup. These tests were performed at the Cytogenetics Laboratory of St Istvan and St Laszlo Hospital and the findings were reported in accordance with the International System for Human Cytogenetic Nomenclature.

The JAK2 haplotype association study included the 124 AML patients of the above cohort who were transplanted in complete remission within the same study period stated above. Allo-HSCT was performed in first complete remission (CR) in 73%, in second CR in 23% and beyond second CR in 4% of the patients. 86% (106/124) of the patients suffered from de novo AML. By conventional karyotyping analysis in 60 cases (48%) chromosomal abnormalities were detected at the diagnosis of AML. The patients in this AML subgroup were slightly younger compared to the whole cohort, the median age at transplantation was 40 years (range 19-62). The median follow up time for surviving patients at last follow up (FU) (80/124, 65%) was 36 months (6-92 months). The donor stem cells originated from a sibling donor in 69 (55.6%) and from an unrelated donor in 55 cases (44.4%). In the vast majority of transplants progenitor cells were collected from peripheral blood (120/124, 96.8%). The cohort was predominantly treated with myeloablative conditioning regimen (104/124, 83.9%), of whom 78 patients had TBI plus cyclophosphamide and 26 a high dose busulfan-based regimen. Reduced intensity conditioning was administered to 16% (20/124) of transplant recipients, who almost exclusively received busulfan/fludarabine (BU/FLU) preparative regimen with anti-thymocyte globulin (ATG). The combination of tacrolimus plus sirolimus was the standard prophylactic regimen for GvHD prevention (n=94, 76%), in ten transplants (8%) other TAC-containing regimens were used, while CSA-based immunosuppression was given to 20 recipients (16%).

Our research was approved by the Hungarian National Ethics Committee and was conducted in accordance with the Helsinki Declaration.

4.2. Transplantation-associated thrombotic microangiopathy

4.2.1. Definition of TA-TMA

The diagnosis of TA-TMA was based on overall TMA criteria including probable TMA cases proposed by Cho et al as summarised in **Table 4.** (156): (1) ≥ 2 schistocytes per high power field; (2) increased serum lactate dehydrogenase; (3) de novo thrombocytopenia (<50 $x10^9$/l) or a decrease in platelet count of ≥50%; (4) reduced haemoglobin; (5) absence of coagulopathy; (6) negative Coombs test. As we aimed at distinguishing more severe cases within the TA-TMA group, we categorised patients as having TA-TMA with organ damage if they fulfilled all criteria of the Blood and Marrow Transplant Clinical Trials Network (BMT CTN) Toxicity Committee Consensus recommendations (151) and showed signs of organ involvement (renal and/or neurological dysfunction).

4.2.2. Management of patients with TA-TMA

Following the diagnosis of TA-TMA the focus of the clinical management was to eliminate the aggravating factors and treat the triggers. As immunosuppressive agents are well-known risk factors for the development of TA-TMA, the regimens were modified upon the suspicion of TA-TMA and CSA, TAC and SIR doses were either reduced or the drugs were completely substituted with alternative immunosuppressive drugs, predominantly with corticosteroids and/or MMF. Other causative factors, such as infections and aGvHD were treated rigorously. As clinical data advocates for poor clinical efficacy with therapeutic plasma exchange (TPE) this modality was not incorporated into the management, and was used only exceptionally. Defibrotide was not part of the treatment strategy and was only administered to patients with concomitant, severe sinusoidal obstructive syndrome (n=3). None of the patients were treated with Eculizumab, which has been proven to have activity in TA-TMA recently, but during the study period its potential benefit was not documented.

4.3. HLA typing

HLA-typing was performed as part of the routine pre-transplant workup at the Transplantation Immunogenetics Laboratory of the Hungarian National Blood Transfusion Service, Budapest, Hungary.

In the sibling donor setting for patients and donors, HLA-A, -B, and -DRB1 typing were performed at low resolution by commercially available sequence-specific primers (SSP, Olerup, Stockholm, Sweden) or sequence-specific oligonucleotide probes (SSOP, One Lambda, Los Angeles, CA, USA). Related donors matched at HLA-A, -B, and -DRB1 antigens were considered as fully matched with the patient.

For patients lacking suitable HLA-matched family members, and for their prospective unrelated donors, high resolution HLA typing was completed by high resolution SSP (Olerup) or sequence-based typing (SBT, Qiagen, ROSE, Valencia, CA, USA) for HLA-A, -B, -C, -DRB1 and partially for HLA-DQB1. In the unrelated setting HLA-A -B, -C and HLA-DRB1 genes were considered for matching between recipient and donor. In case recipients and donors were matched for all four genes, HLA matching was regarded as full (8/8, considering two copies of each gene), in case of any mismatch, recipient/donor pairs were regarded as HLA mismatched (less than 8/8 match).

With relevance to the TA-TMA study in the whole cohort, 2 out of 425 patients underwent a transplant from a HLA-DRB1 mismatched donor at high resolution. Both patients and their donors carried HLA-DRB1*11, however not the same allele. Giving that our study investigated the association of TA-TMA with HLA-DRB1*11 allele group and not the distinct alleles, these two cases were not excluded from the analyses.

In the JAK2 46/1 haplotype association work, 14 patients received grafts from HLA-mismatched donors considering HLA-A, -B, -C, -DRB1 antigen matching. Five patients underwent a procedure from a HLA-A, one from a HLA-B and eight from a HLA-C mismatched donor, making the study population ideal for the investigation of the role of non-HLA genes in association with post-transplantation complications.

4.4. Molecular genetic methods

For the genetic studies whole genomic DNA was isolated from peripheral blood or bone marrow with Puregene Gentra DNA Isolation kit.

We used the detection of SNP rs12343867, a fully-linked, tagging SNP to JAK2 haplotype for identification of the haplotype. As JAK2 rs12343867_C tags 46/1 haplotype (the nucleotide C represents the haplotype, while T determines non-46/1 haplotype), JAK2 rs12343867_C allele frequencies are presented as 46/1 haplotype frequencies.

LightCycler technology (LightCycler 480II, Roche Diagnostics, Basel, Switzerland) employing melting curve analysis with hybridization probe detection format was performed to identify the alleles of *JAK2* gene intron 14 SNP, rs12343867 (HGVS. names: NM_004972.2: c.1864+404A>T, NT_008413.17: g.5064189T>C). Amplification primers (JAK2-LCF, JAK2-LCR) and hybridization probes (JAK2-SENS, JAK2-ANC) designed by LightCycler Probe Design software (Roche Diagnostics, Basel, Switzerland) were the following: JAK2-LCF: 5'-GCG GGT AGG ACT ATT CAG TTA TAT CTT G-3', JAK2-LCR: 5'-CTG TAT AGT ATT AAA GCA TGG GGT ACG A-3', JAK2-SENS: 5'-AGA AAT GAT TAC GTT GAT ATG ATA CTA GA-Fluorescein-3', JAK2-ANC: 5'-LC Red 640-TAT TTT TTG GCT AAA TTT AGG TGT TCA CAG AAA CTA CTA A-P-3'. Oligonucleotides were diluted to 10 pmol/µl. Asymmetric polymerase chain reaction (PCR) was performed with 0.125 µl forward (JAK2-617F-forw or JAK2-617V-forw or JAK2-LCF) and 0.625 µl reverse (JAK2-LCR) amplification oligonucleotides (1:5 forward to reverse primer ratio), 0.25 µl of labelled oligonucleotides (SENS and ANC), 50 ng genomic DNA, 2 x PCR Master Mix (MyTaq, catalogue number: MTX-313211) in a reaction volume of 20 µl. Cycling conditions were as follows: initial denaturation at 95°C for 3 min, followed by 65 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s. After amplification a melting curve analysis was performed by cooling the samples to 40°C, then gradually heating them to 80°C with a ramp rate of 0.11 °C/s. The decline of fluorescence was continuously monitored. Melting curves were converted to melting peaks with wild-type and variant alleles showing distinct melting points. Results were evaluated by two independent investigators.

4.5. Statistical analysis

Categorical variables were compared using the Chi-square or the Fisher's exact tests, while continuous variables were tested by Mann-Whitney or Kruskal-Wallis tests. Binary logistic regression analysis was conducted to determine Odds ratios (OR) for TA-TMA and aGvHD development. A dominant model was applied for the statistical studies on 46/1 haplotype; whereby comparisons were made between the following two groups: people with TT, non-46/1 haplotype genotype versus individuals with at least one minor allele, either with heterozygous TC or homozygous CC genotype. Cumulative incidences were calculated by the Fine and Gray model including death as competing risk in the analysis (226). Overall survival (OS) was calculated from the day of transplant until the date of death from any cause or last follow-up. Survival between subgroups were compared by the log-rank test and estimated by the Kaplan-Meier method. Cox regression analysis was applied for studying the effect of independent risk factors on survival, hazard ratios (HR) and 95% confidence intervals (95% CI) were calculated. The analyses were two-tailed and p values less than 0.05 were considered statistically significant, whereas p values between 0.05 and 0.1 were referred to as tendency. The statistical analyses were performed by SPSS Statistics software version 22 (Armonk, NY, USA) and STATA10 software (College Station, TX, USA).

5. RESULTS

5.1. TA-TMA STUDY

5.1.1. Patient and transplantation characteristics and distribution of HLA-DRB1*11

HLA-DRB1*11 was the most frequent investigated HLA class II antigen in the entire HSCT cohort with a carrier frequency of 32.2% (127 heterozygous and 10 homozygous recipients). Of note HLA-DPB1 testing was not part of the routine practice at the time of our study and HLA-DQB1 results were not available for all transplant recipients, therefore we considered HLA-A-, B-, C-, and DRB1 only (8/8 match). The HLA-DRB1*11 carrier frequency was comparable to that of the data from the Hungarian National Stem Cell Donor Registry (28.2%). Patient and transplantation characteristics and their distribution according to HLA-DRB1*11 for the entire cohort are presented in **Table 5**. The baseline characteristics did not vary in HLA-DRB1*11 carriers vs. non-carriers except for donor type (HLA-DRB1*11 carrier frequency in HSCT with unrelated donor: 38.9%, while in HSCT with sibling donor: 25.8%, p=0.005, **Table 5.**). The HLA-DRB1*11 carrier frequency among patients transplanted with unrelated donors was significantly higher in comparison with the Hungarian registry data (p=0.001), while in the sibling setting there was no difference (p=0.5). Due to the differential distribution additional analyses were done depending on donor type.

Table 5. Patient and transplantation-related characteristics in the entire cohort, in HLA-DRB1*11 carrier and non-carrier patients.

Variable	Total number	HLA-DRB1*11 positive patients		HLA-DRB1*11 negative patients		P
	n	n	%	n	%	
Entire cohort	425	137	32.2	288	67.8	
Age median (range)	43 (19-73)	42	19-67	43	19-73	0.605
Gender (female/male)	186/239	59/78	31.7/32.6	127/161	68.3/67.4	0.917
Recipient/Donor gender						1
Male recipient/Female donor	103	33	32	70	68	
Others	322	104	32.3	218	67.7	
Diagnosis						0.342
AML	157	45	28.7	112	71.3	
ALL	75	23	30.7	52	69.3	
Other myeloid	82	33	40.2	49	59.8	
Other lymphoid	111	36	32.4	75	67.6	
Stem cell source						1
Peripheral blood/other	414/11	134/3	32.4/27.3	280/8	67.6/72.7	
Donor						**0.005**
Sibling	217	56	25.8	161	74.2	
Unrelated	208	81	39	127	61	
Conditioning intensity						1
MAC	267	86	32.2	181	67.8	
RIC	158	51	32.3	107	67.7	
MAC regimen						0.71
TBI + CY	157	52	33.1	105	66.9	
TBI + VP16 or MEL	51	14	27.5	37	72.5	
High dose BU-containing	59	20	33.9	39	66.1	
RIC regimen						0.75
BU + FLU	90	29	32.2	61	67.8	
Others	68	22	32.4	46	67.6	
GvHD prophylaxis						0.143
CSA +/- MMF or MTX	100	26	26	74	74	
TAC + SIR or MMF or MTX	325	111	34.2	214	65.8	
Acute GvHD						0.737
No and grade I	296	97	32.8	199	67.2	
Grade II-IV	129	40	31	89	69	
CMV reactivation/disease						0.427
Yes	80	29	36.2	51	63.8	
No	345	108	31.3	237	68.7	
ABO compatibility						0.836
ABO compatible	201	64	31.8	137	68.2	
ABO incompatible	222	73	32.9	149	67.1	

P values indicate comparisons between HLA-DRB1*11 positive and negative groups. **Abbreviations for Tables 5 and 6.** ABO=ABO blood group, ALL=acute lymphoblastic leukaemia, AML=acute myeloid leukaemia, BU=busulfan, CMV=cytomegalovirus, CSA=cyclosporine, CY=cyclophosphamide, FLU=fludarabine, GvHD=graft-versus-host disease, HLA=human leucocyte antigen, HSCT=haematopoietic stem cell transplantation, MAC=myeloablative conditioning, MEL=melphalan, MMF=mycophenolate mofetil, MTX=methotrexate, NA=not applicable, P=probability value, RIC=reduced intensity conditioning, SIR=sirolimus, TAC=tacrolimus, TA-TMA=transplantation-associated thrombotic microangiapathy, TBI=total body irradiation, VP16=etoposide

5.1.2. Predicting factors for the development of TA-TMA

TA-TMA was diagnosed in 18.8% of the patients (80/425) (**Table 6.**), among whom 41 (51.3% of TA-TMA patients) developed organ damage. The median time to TA-TMA manifestation from the day of transplant was 40 days (range 6-1670 days) and in the vast majority of patients (81%) TA-TMA presented within the first 100 days of transplantation.

As a first step in the series of analyses, clinical factors previously described in association with TA-TMA were tested and the associations are showed in **Table 6**.

Table 6. Patient and transplantation-related characteristics in the entire cohort, in HLA-DRB1*11 carriers and non-carriers.

Variable	TA-TMA in the entire HSCT cohort			TA-TMA in HLA-DRB1*11 carriers			TA-TMA in non-HLA-DRB1*11 carriers			P
	n1	n2	%	n1	n2	%	n1	n2	%	
Entire HSCT cohort	80	425	18.8	34	137	24.8	46	288	16.0	
Age										0.622
Below median	42	212	19.8	21	71	29.6	21	141	14.9	
Above median	38	213	17.8	13	66	19.7	25	147	17.0	
Patient gender										0.708
Female	33	186	17.7	15	59	25.4	18	127	14.2	
Male	47	239	19.7	19	78	24.4	28	161	17.4	
Recipient/Donor gender										0.386
Male/Female	16	103	15.5	10	33	30.3	6	70	8.6	
Others	64	322	19.9	24	104	23.1	40	218	18.3	
Donor type										**<0.001**
Sibling	23	217	10.6	10	56	17.9	13	161	8.1	
Unrelated	57	208	27.4	24	81	29.6	33	127	26.0	
Conditioning intensity										**0.003**
MAC	62	267	23.2	26	86	30.2	36	181	19.9	
RIC	18	158	11.4	8	51	15.7	10	107	9.3	
GvHD prophylaxis										**0.003**
CSA-based	9	100	9.0	3	26	11.5	6	74	8.1	
TAC-based	71	325	21.8	31	111	27.9	40	214	18.7	
aGvHD										**<0.001**
No and grade I	31	296	10.5	12	97	12.4	19	199	9.5	
Grade II-IV	49	129	38.0	22	40	55.0	27	89	30.3	
CMV reactivation/disease										**0.003**
Yes	25	80	31.3	12	29	41.4	13	51	25.5	
No	55	345	16.0	22	108	20.4	33	237	14.0	
ABO matching										0.620
ABO compatible	40	200	20.0	20	64	31.3	20	136	14.7	
ABO incompatible	40	223	17.9	14	73	19.2	26	150	17.3	
HLA-DRB1*11 status										**0.034**
Non-carrier	46	288	16.0		NA			NA		
Carrier	34	137	24.8							

Affected individuals (n1) from the cohorts (n2), and percentages (%) are listed in the table. P-values below 0.05 (shown in boldface character) indicate significant associations of TA-TMA development with the respective variables.

TA-TMA affected 27.4% of patients with an unrelated donor as opposed to only 10.6% in those with a sibling donor ($p<0.001$). Similarly, the incidence of TA-TMA was significantly higher following the administration of myeloablative conditioning compared to reduced intensity conditioning (23.2% vs. 11.4%, $p=0.003$). Especially high frequency (32.2%) was observed when high-dose busulfan was part of the myeloablative conditioning regimen ($p=0.007$ and $p=0.08$, when compared to all other regimens and other MAC regimens, respectively). TA-TMA was diagnosed in 9.0% in the CSA-based aGvHD prophylactic subgroup, whereas the incidence increased with TAC administration (21.8%, $p=0.003$), irrespectively of the other agent given in combination with TAC. Adding sirolimus to TAC instead of mycofenolate mofetil or methotrexate did not add to the risk (22.1% vs. 21.0%), but TA-TMA developed slightly earlier with a median of 32 days versus 47 days ($p=0.14$). Among the 80 TA-TMA patients, 66 simultaneously presented with aGvHD during follow-up, and in 90.9% of these cases aGvHD preceded TA-TMA. While TA-TMA was rare in patients without aGvHD (7.8%), 34.9% and 55.0% were diagnosed among those who had a grade II-III or IV aGvHD, respectively. CMV reactivation/disease were also proved to be risk factors for TA-TMA ($p=0.003$). Other factors such as age, recipient's gender, recipient and donor gender matching, diagnosis and ABO compatibility were not associated with the development of TA-TMA.

In addition to the above mentioned, well-documented risk factors, analyses confirmed that carrying HLA-DRB1*11 was also significantly associated with the manifestation of TA-TMA. It occurred in 24.8% (n=34) of HLA-DRB1*11 positive versus in 16.0% (n=46) of HLA-DRB1*11 negative patients ($p=0.034$ in univariate analyses). The development of organ damage was not statistically different in HLA-DRB1*11 carriers (44%; 15/34) and in non-carriers (56%; 26/46; $p=0.366$). When death was included in the analyses as a competing event for the manifestation of TA-TMA, the cumulative incidence of TA-TMA showed significant alteration according to HLA-DRB1*11 ($p=0.026$, **Figure 2.**).

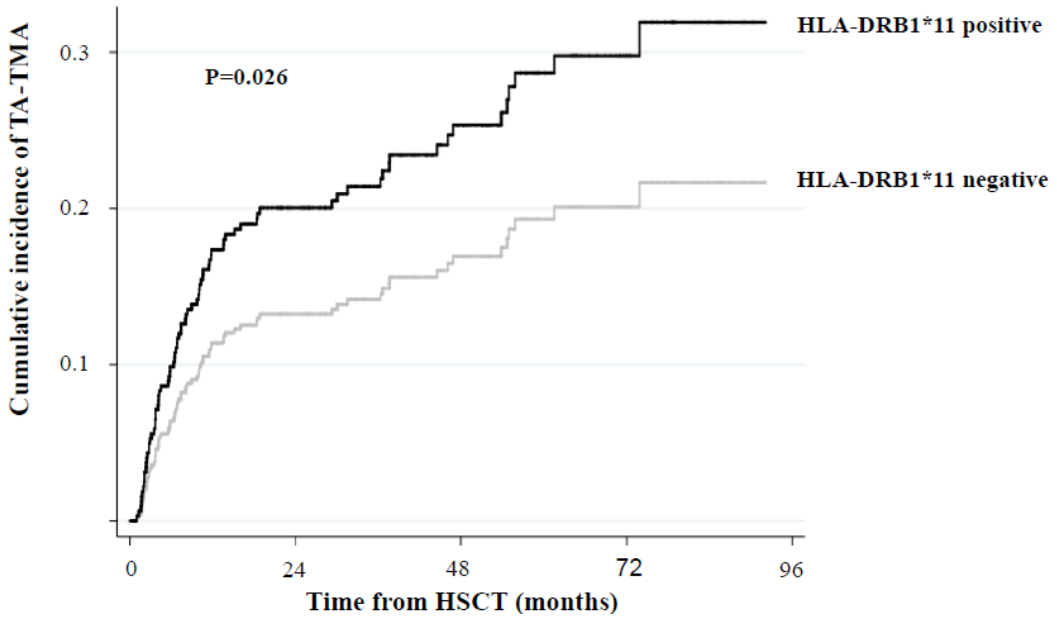

Figure 2. Cumulative incidence of TA-TMA in HLA-DRB1*11 positive and negative HSCT patients.

Abbreviations for Figures 2-6. HLA=human leucocyte antigen, HSCT=haematopoietic stem cell transplantation, P=probability value, TA-TMA=transplantation-associated thrombotic microangiopathy.

The distribution of the identified clinical risk factors for the development of TA-TMA (donor type, conditioning intensity, CMV, aGVHD, immunosuppression, HLA-DRB1*11) did not differ in patients with and without signs of organ damage (data not shown).

Factors with a p value below 0.1 in univariate analyses were included in the multivariate analysis. Of these parameters the association of TMA with donor type and grades II-IV aGvHD was independently significant, there was a tendency with relation to CMV and HLA-DRB1*11, while the other parameters failed to show independent association (**Table 7.**).

Table 7. Multivariate analyses of factors influencing TA-TMA development

Variables	OR	95% CI lower value	95% CI upper value	P
Donor type (MUD vs. sibling)	1.934	1.078	3.468	0.027
Conditioning intensity (MAC vs. RIC)	1.691	0.86	3.326	0.128
GvHD prophylaxis (TAC vs. CSA)	1.632	0.686	3.885	0.268
GvHD (grade II-IV vs. grade 0-I)	4.134	2.393	7.141	<0.001
CMV (reactivation/disease vs. not)	1.776	0.963	3.276	0.066
HLA-DRB1*11 (carrier vs. non-carrier)	1.664	0.959	2.89	0.070

Abbreviations for Table 7. CI=confidence interval, CMV=cytomegalovirus, GvHD=graft-versus-host disease, HLA=human leucocyte antigen, MAC=myeloablative conditioning, MUD=matched unrelated donor, OR=odds ratio, P=probability value, RIC=reduced intensity conditioning, TAC=tacrolimus.

Separate analyses performed due to uneven distribution of donor type among HLA-DRB1*11 carriers and non-carriers found that HLA-DRB1*11 carriership was significantly associated with the emergence of TA-TMA in the sibling subgroup (17.8% [10/56] in HLA-DRB1*11 positive versus 8.1% [13/161] in HLA-DRB1*11 negative cases; p=0.047), but not in the unrelated donor subset (29.6% [24/81] in HLA-DRB1*11 positive versus 26.0% [33/127] in HLA-DRB1*11 negative cases; p=0.633).

5.1.3. Predicting factors for the survival of TA-TMA patients

For alive patients at last follow-up the median follow-up time was 36 months (range 6-92 months). The overall survival of patients who developed TA-TMA was significantly worse compared to patients without the complication (**Figure 3.,** A vs. B, p<0.001, 36-month OS: 27.4±5.3% vs. 55.3±2.9%). Multivariate analyses confirmed that TA-TMA was an independent adverse risk factor for survival (HR 1.9, 95% CI: 1.35-2.62), considering recipient age at transplantation, donor type and severe aGvHD (grades III-IV) as covariates.

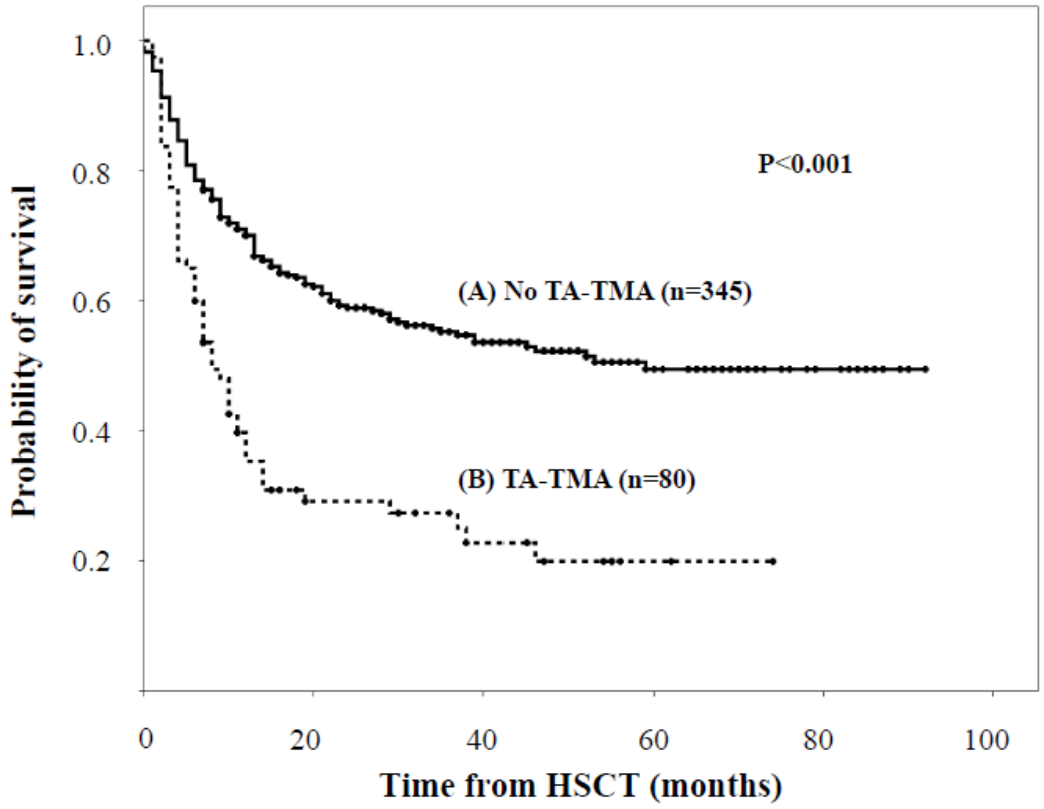

Figure 3. Kaplan-Meier analysis of post-transplantation overall survival.

Post-transplantation OS was inferior in patients with TA-TMA compared with patients without the complication. (A vs. B, p<0.001).

Within the TA-TMA group, the outcome of patients with organ damage was even more unfavourable in contrast to individuals without organ involvement (B vs. C, p=0.025, **Figure 4.**).

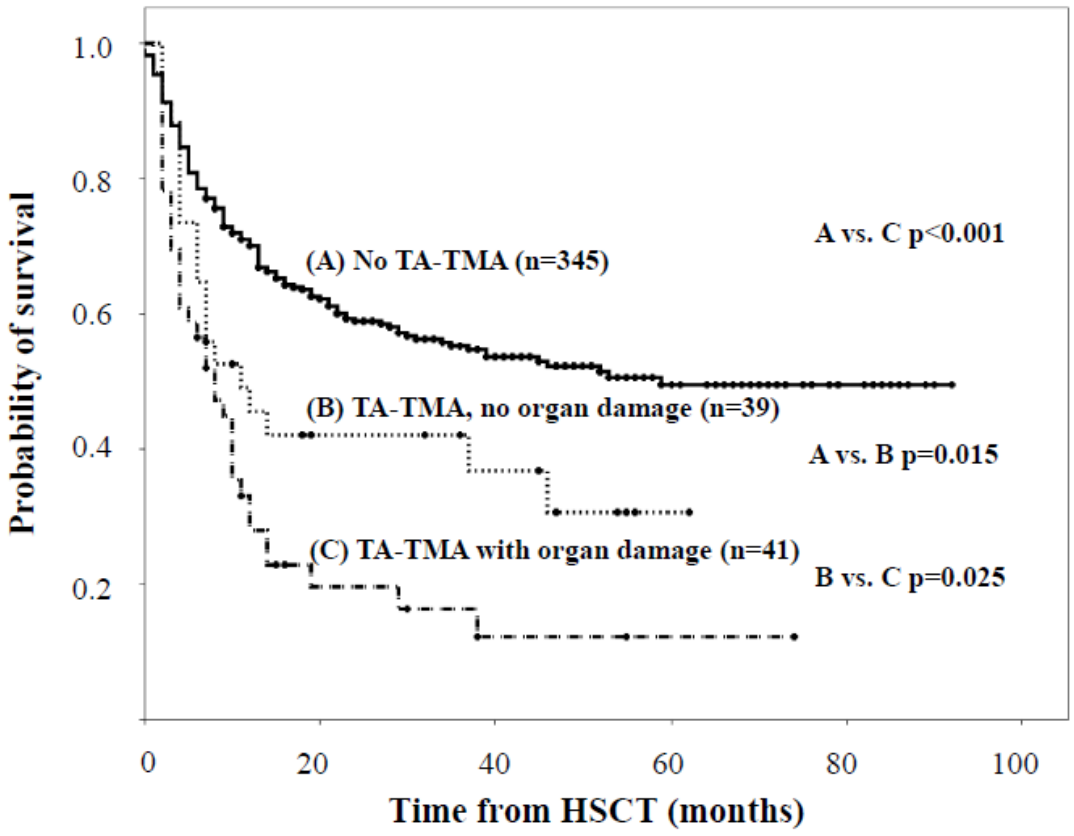

Figure 4. Post-transplantation OS among TA-TMA patients with and without organ damage and patients without TA-TMA.

The survival of TA-TMA affected individuals differed according to the presence of organ damage (B vs. C, p=0.025). The survival of patients without TA-TMA was significantly better compared to both subgroups, patients with TA-TMA without organ involvement (A vs. B, p=0.015) and patients with TA-TMA with organ damage (A vs. C, p<0.001).

Interestingly, although the survival of patients not suffering from TA-TMA was not influenced by HLA-DRB1*11, carriers of HLA-DRB1*11 alleles displayed superior OS within the TA-TMA cohort (C vs. D, p=0.003, **Figure 5.**).

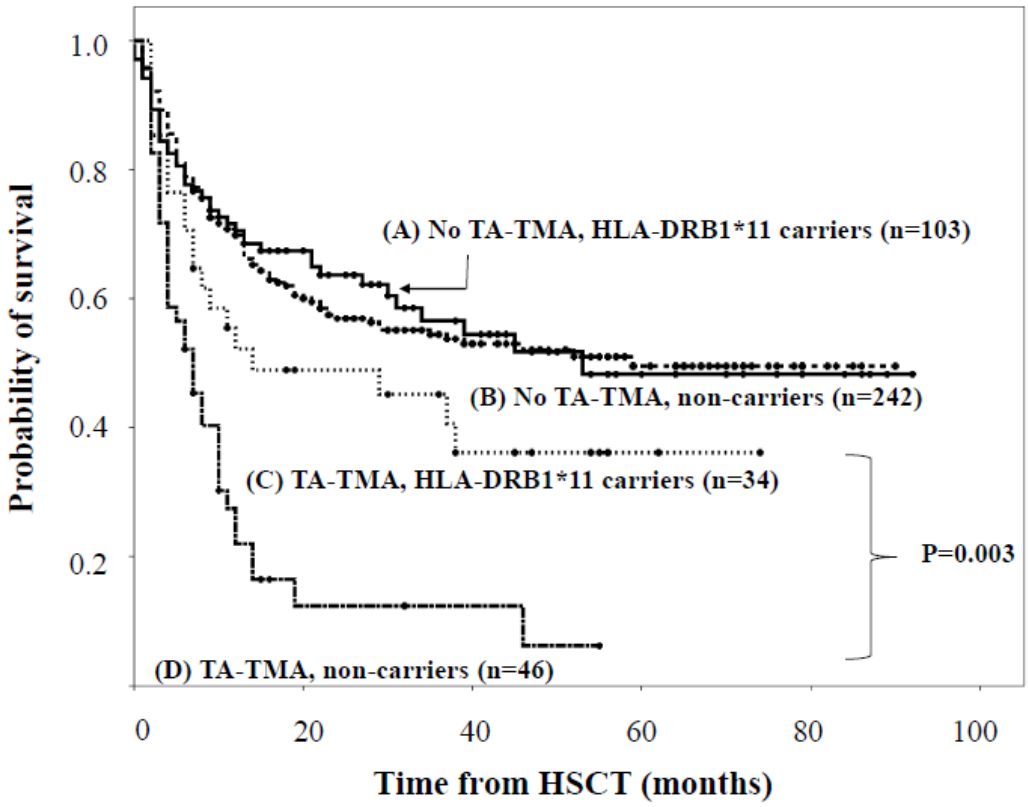

Figure 5. Post-transplantation OS in patients with and without TA-TMA according to HLA-DRB1*11 status.

The survival was not different in patients who did not suffer from TA-TMA depending on HLA-DRB1*11 carrier status (A vs. B, upper two curves, p=0.778). In contrast, the survival of patients with TA-TMA was significantly better in HLA-DRB1*11 carriers compared to non-carriers (C vs. D, lower two curves, p=0.003).

Among TA-TMA patients carriership of HLA-DRB1*11 was an independent predictor of survival (HR: 0.46, 95% CI: 0.27-0.81, p=0.007), together with grades III-IV aGvHD (HR: 1.8, 95% CI: 1.07-3.14, p=0.027). Further assessing the role of HLA-DRB1*11 on survival, we noticed that in patients who developed TA-TMA with organ damage, there was only a tendency for superior survival among HLA-DRB1*11 carriers (C vs. E, p=0.076, **Figure 6.**), while the survival difference was marked for those patients, who suffered from a milder form of TA-TMA without organ involvement (A vs. D, p=0.027).

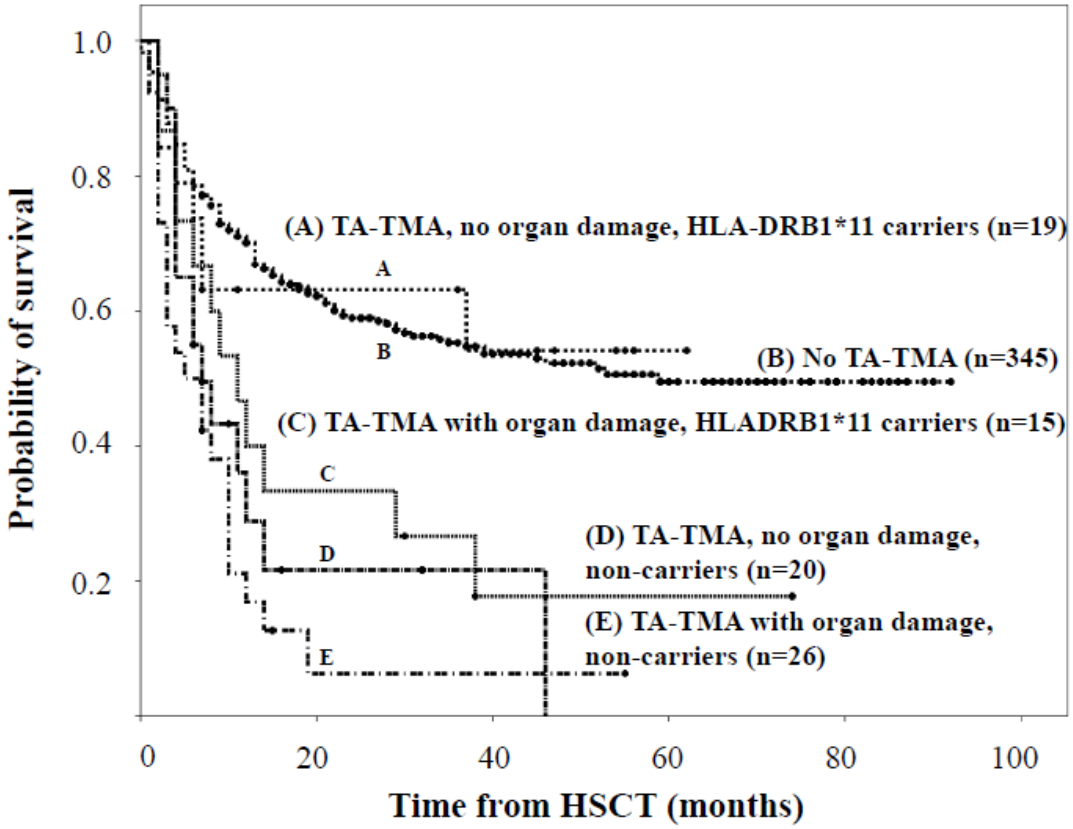

Figure 6. Post-transplantation OS in TA-TMA patients with and without organ damage according to HLA-DRB1*11 status.

The survival difference was marked among TA-TMA affected patients with no organ damage depending on HLA-DRB1*11 carrier status (A vs. D p=0.027). The survival of HLA-DRB1*11 carriers with TA-TMA without organ damage was comparable to patients without TA-TMA (A vs. B p=0.962, global p<0.001).

5.2. JAK2 46/1 HAPLOTYPE STUDY

5.2.1. HSCT characteristics and outcome according to patients' JAK2 46/1 haplotype

In this study a subgroup of 124 patients were included who suffered from AML and were transplanted in CR. Among the 124 transplant recipients, 64 (51.6%) carried the TT, non-46/1 haplotype genotype, the number of heterozygous and homozygous carriers for the 46/1 haplotype was 49 (39.5%) and 11 (8.9%), respectively. The calculated allele frequency of the 46/1 haplotype was 29±6% (percentage and 95% confidence interval). Dominant model was applied in our calculation as hetero- and homozygous individuals were grouped together as 46/1 carriers.

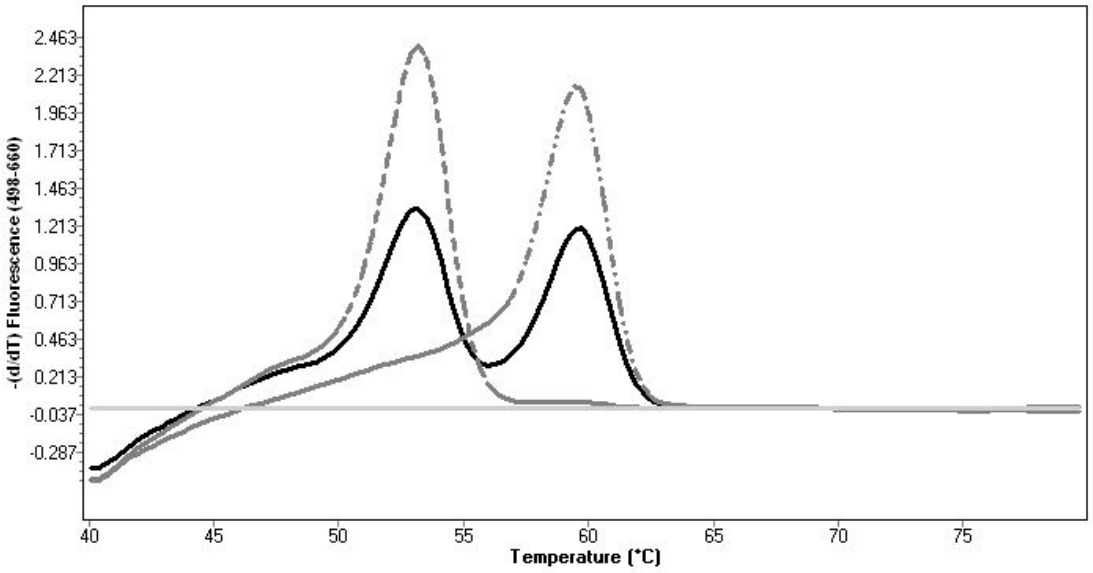

Figure 7. JAK2 46/1 genotyping with melting curve analyses performed on LC480 instrument.

A single nucleotide change results in 6◦C temperature shift in the presence of the genetic variants. Wild type (dashed line on the left, melting temperature at 53◦C), heterozygous (continuous line with two peaks) and homozygous (dotted and dashed line on the right, melting temperature at 59◦C) 46/1 haplotype carriers can be distinguished according to shifted melting temperatures.

The baseline characteristics, such as gender, type of AML, karyotype, donor type, conditioning intensity, GvHD preventative regimens, were evenly distributed in patients with non-46/1 and 46/1 haplotype (**Table 8.**).

Table 8. Baseline characteristics and outcome data according to recipient 46/1 haplotype.

Variable	Total cohort		46/1 non-carrier		46/1 carrier		P
	n	%	n	%	N	%	
Entire AML cohort	124	100	64	51.6	60	48.4	
Age at HSCT (years)	40	19-62	42	19-62	40	21-62	0.64
Origin							1.0
De novo AML	106	85.5	55	85.9	51	85.0	
Not de novo AML	18	14.5	9	14.1	9	15.0	
Karyotype							0.66
Normal	61	49.2	30	46.9	31	51.7	
Abnormal	60	48.4	33	51.6	27	45.0	
Recipient gender							0.86
Female	61	49.2	32	50.0	29	48.3	
Male	63	50.8	32	50.0	31	51.7	
Donor							0.59
Sibling	69	55.6	34	53.1	35	58.3	
Unrelated	55	44.4	30	46.9	25	41.7	
HLA-matching							0.58
8/8 matched	110	88.7	58	90.6	52	86.7	
Mismatched (<8/8)	14	11.3	6	9.4	8	13.3	
Conditioning intensity							1.0
MAC	104	83.9	54	84.4	50	83.3	
RIC	20	16.1	10	15.6	10	16.7	
aGvHD prophylaxis							0.79
CSA +/- MMF or MTX	20	16.1	11	17.2	9	15.0	
TAC + MMF or MTX	10	8.1	6	9.4	4	6.7	
TAC+SIR	94	75.8	47	73.4	47	78.3	
Acute GvHD							**0.006**
No and grade I	87	70.2	52	81.2	35	58.3	
Grade II-IV	37	29.8	12	18.8	25	41.7	
Onset of aGvHD II-IV (days)	28	3-120	20	3-80	40	7-120	0.25
Relapse after allo-HSCT							**0.004**
No	106	85.5	49	76.6	57	95.0	
Yes	18	14.5	15	23.4	3	5.0	
Time to relapse (months)	5	1-29	5	1-29	7	4-9	0.91
Cause of death							**0.024**
Relapse	14	31.8	11	47.8	3	14.3	
TRM	30	68.2	12	52.2	18	85.7	
Time to death (months)	6.5	2-46	8	2-29	6	2-46	0.80

Time variables are expressed as median and range. **Abbreviations for Tables 8 and 9.** aGvHD=acute graft-versus-host disease, allo-HSCT= allogeneic haematopoietic stem cell

transplantation, AML=acute myeloid leukaemia, CSA=cyclosporine, GvHD=graft-versus-host disease, HLA=human leucocyte antigen, HSCT=haematopoietic stem cell transplantation, MAC=myeloablative conditioning, MEL=melphalan, MMF= mycophenolate mofetil, MTX=methotrexate, P=probability value, RIC=reduced intensity conditioning, SIR=sirolimus, TAC=tacrolimus, TRM=transplant-related mortality.

Engraftment did not differ depending on the haplotype and the median time to neutrophil engraftment (defined as the first day of three consecutive days with an absolute neutrophil count > $0.5x10^9$/l) was the same in haplotype carriers and non-carriers (15 days in both groups, range 11-36 in non-carriers and 9-42 in carriers, p=0.5).

Acute GvHD grades II-IV developed in 29.8% of the patients and incidences did not show alteration in patients with normal karyotype AML vs. in the presence of karyotypic abnormalities (19/61, 31.1% in normal vs.. 18/60, 30.0% in abnormal karyotype, p=1). In 46/1 haplotype carriers we observed significantly more grades II-IV aGvHD compared with non-carriers (**Table 8.**). By 100 days post-transplant aGvHD grades II-IV affected 41.7% of haplotype carriers as opposed to 18.8% of non-carriers (p=0.008, **Figure 8.**). Separate analysis for gastrointestinal (GI) aGvHD confirmed similar figures showing that GI aGvHD developed more frequently in 46/1 haplotype carriers (13/60, 21.7% vs. 4/64, 6.3%, p=0.018). Although the sample size of the homozygous haplotype carrier subgroup was low, of note six out of eleven patients developed GII-IV aGvHD (54.5%).

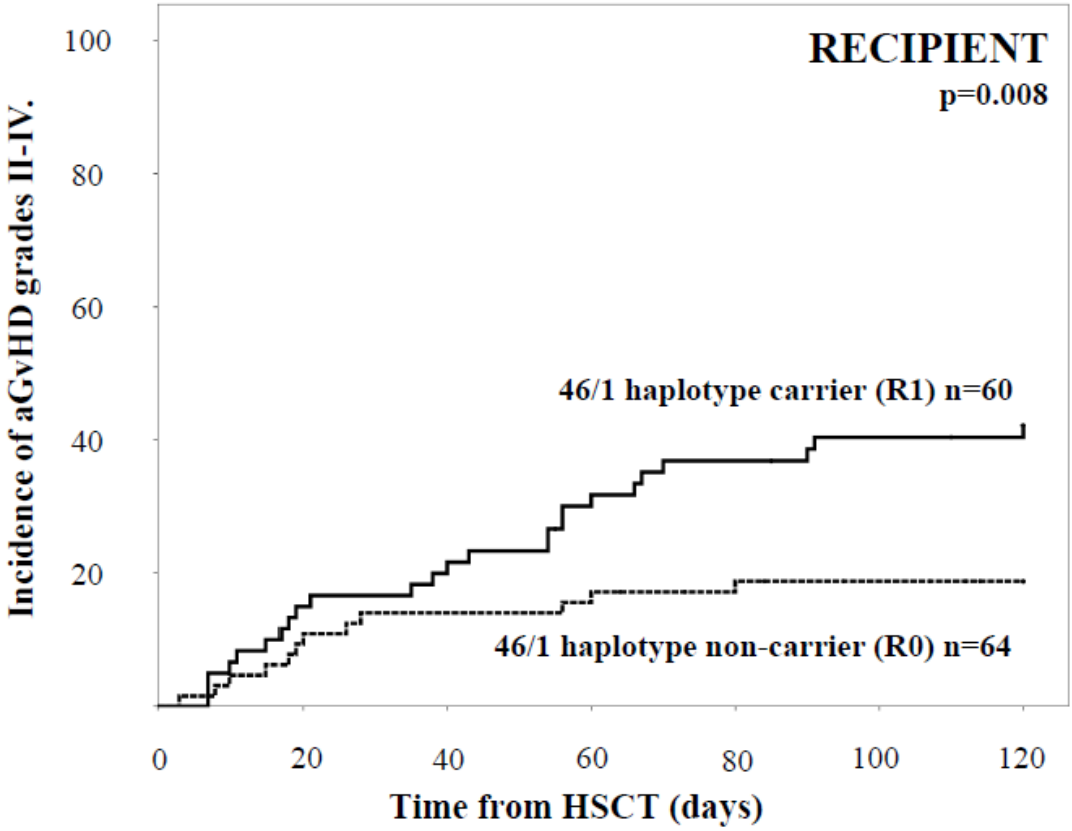

Figure 8. Incidence of aGvHD grades II-IV by 100 days according to recipient haplotype. Incidence was 41.7% in haplotype carriers (R1) in contrast to 18.8% in non-carriers (R0, p=0.008).

Abbreviations for Figure 8. aGvHD=acute graft-versus-host disease, HSCT= haematopoietic stem cell transplantation, P=probability value, R0=recipient not carrying JAK2 46/1 haplotype, R1=JAK2 46/1 haplotype carrier recipient.

We assessed whether the association of aGvHD with the haplotype was observed in all GvHD prophylactic regimen subgroups and we detected that, in the largest group treated with TAC/SIR (n=94, 75.8%) the correlation remained significant (23/47, 48.9% aGvHD grades II-IV in 46/1 haplotype vs. 8/47, 17.0% in non-46/1 haplotype, p=0.002), but similar association did not apply for the remaining 30 patients (2/13, 15.4% vs. 4/17, 23.5%, p=0.672).

Until last follow up 18 (14.5%) patients relapsed at a median of 5 months (range 1-29 months) following allo-HSCT, of whom 15 (83.3%) were non-46/1 haplotype carriers and only 3 (16.7%) carriers (p=0.004). Taking death from any cause as a competing risk into consideration the cumulative incidence of relapse at 2 years differed significantly in haplotype carriers (5.0%) in comparison with non-carriers (23.5%, p=0.008).

The 2-year OS of the cohort was 65.4±4.5%. The cause of death among the deceased patients (44/124) showed variation in haplotype carriers vs. non-carriers. In 46/1 haplotype carriers the major causes of death were transplantation-related complications, including GvHD (18/21, 85.7%), with only 3 deaths (14.3%) attributable to relapse. There was no relapse in homozygous carriers. In contrast almost half of the non-46/1 haplotype carriers (11/23, 47.8%) died of relapsing AML, and non-relapse mortality was relatively lower accounting for 52.2% (p=0.024).

Despite the differences in the causes of death and relapse rate according to the haplotype, OS was comparable in 46/1 haplotype carriers and non-carriers (2-year OS 69.2±6.1% in carriers vs. 60.7±6.7% in non-carriers, p=0.732).

5.2.2. HSCT characteristics and outcome according to donors' JAK2 46/1 haplotype

The distribution of donor JAK2 genotype was very similar to that of the recipients'; TT genotype was identified in 66 (53.2%), TC in 45 (36.3%) and CC in 13 (10.5%) cases. Correspondingly to recipient data, the baseline transplantation characteristics did not differ in HSCT with 46/1 and non-46/1 haplotype carrier donors, but aGvHD grades II-IV was more prevalent in patients who were transplanted from a donor with 46/1 haplotype (**Table 9.**).

Table 9. Baseline characteristics according to donor 46/1 haplotype.

Variable	46/1 non-carrier		46/1 carrier		P
	n	%	n	%	
Entire AML cohort	66	53.2	58	46.8	
Origin					0.45
De novo AML	58	87.9	48	82.8	
Not de novo	8	12.1	10	17.2	
Karyotype					0.89
Normal	32	48.5	29	50	
Abnormal	32	48.5	28	48.3	
Recipient gender					0.59
Female	34	51.5	27	46.6	
Male	32	48.5	31	53.4	
Donor					0.59
Sibling	35	53.0	34	58.6	
Unrelated	31	47.0	24	41.4	
Conditioning intensity					0.63
MAC	54	81.8	50	86.2	
RIC	12	18.2	8	13.8	
aGvHD prophylaxis					0.59
CSA +/- MMF or MTX	12	18.2	8	13.8	
TAC + MMF or MTX	4	6.0	6	10.3	
TAC+SIR	50	75.8	44	75.9	
Acute GvHD					**0.031**
No and grade I	52	78.8	35	60.3	
Grade II-IV	14	21.2	23	39.7	
Relapse					1
No	56	84.8	50	86.2	
Yes	10	15.2	8	13.8	
Cause of death					**0.049**
Relapse	9	50.0	5	19.2	
TRM	9	50.0	21	80.8	

Moderate and severe aGvHD was diagnosed in 39.7% vs. 21.2% of patients with haplotype carrier vs. non-carrier donors by day 100 (p=0.038, **Figure 9.**). Alike, gastrointestinal aGVHD developed more frequently in patients with haplotype carrier donors (12/58, 20.7% vs. 5/66, 7.6%, p=0.04). Five of thirteen patients (38.5%) transplanted from a donor homozygous for the haplotype suffered from aGvHD GII-IV.

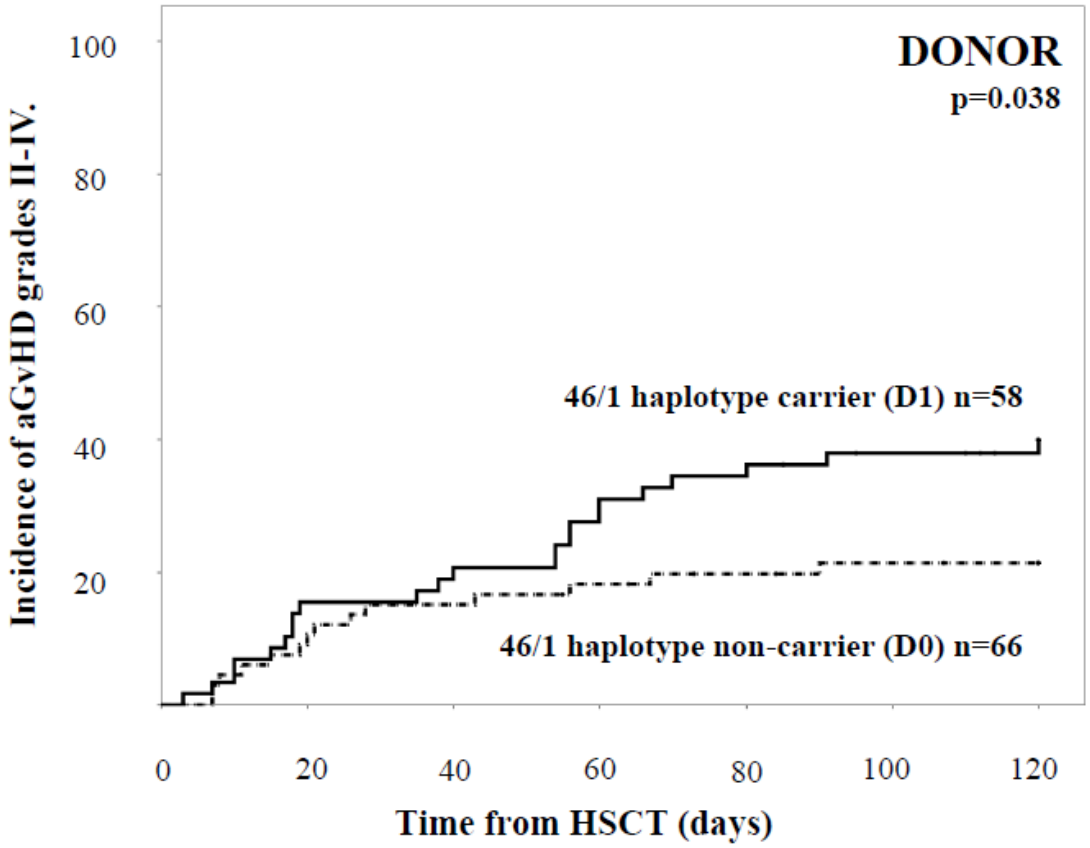

Figure 9. Incidence of aGvHD grades II-IV depending on JAK2 46/1 haplotype of the donor. Acute GvHD grades II-IV affected 39.7% vs. 21.2% of patients with haplotype carrier (D1) vs. non-carrier donors (D0, p=0.038) by day 100.

Abbreviations for Figure 9. aGvHD=acute graft-versus-host disease, HSCT= haematopoietic stem cell transplantation, D0=donor not carrying JAK2 46/1 haplotype, D1=JAK2 46/1 haplotype carrier donor, P=probability value.

Among the 44 deceased patients we found that patients more likely died of transplantation related complications if their donors were 46/1 carriers (21/26, 80.8% vs. 9/18, 50.0%, p=0.049). As opposed to the observed association between recipient haplotype and relapse rate, similar correlation lacked for the donor haplotype and the relapse rates were comparable in patients transplanted from carrier and non-carrier donors (8/58, 13.8% vs. 10/66, 15.2%, p=1). Only one patient relapsed among those, whose donors were homozygous haplotype carriers (7.7%). Similarly to survival figures in relation to the recipient haplotype, the survival was not significantly different according to the donor

haplotype, however there was a tendency for better OS in patients transplanted from non-46/1 haplotype carriers vs. 46/1 haplotype carriers: 2-year OS 73.9±5.7 vs. 56.0±6.9% (p=0.056).

5.2.3. Impact of the recipient-donor JAK2 46/1 haplotype combinations

Initiated by the above results suggesting that both the recipient and the donor 46/1 haplotype correlated with the development of aGvHD, we performed further analyses to investigate whether the associations were independent. In univariate analyses, aGvHD grades II-IV were significantly associated with unrelated donor type (p=0.031), myeloablative conditioning (p=0.036), recipient age at transplantation (p=0.046), JAK2 46/1 haplotype in the recipient (p=0.006) and in the donor (p=0.031). Multivariate analyses confirmed that recipient JAK2 46/1 haplotype was independently associated with aGVHD (p=0.012), whereas the donor JAK2 haplotype was not (p=0.08) (**Table 10.**).

Table 10. Multivariate analyses of factors influencing aGvHD grades II-IV development.

Variables	**OR**	**95% CI**	**P**
Age	0.982	0.943- 1.023	0.382
Donor type (unrelated vs. sibling)	3.544	1.433- 8.763	0.006
Conditioning intensity (MAC vs. RIC)	4.930	0.896- 27.130	0.067
Recipient JAK2 46/1 haplotype	3.181	1.284- 7.879	0.012
Donor JAK2 46/1 haplotype	2.232	0.908- 5.489	0.080

Factors with a p value below 0.1 in univariate analyses were included in the multivariate analysis.
Abbreviations for Table 10. CI=confidence interval, JAK2=Janus kinase 2, MAC=myeloablative conditioning, OR=odds ratio, P=probability value, RIC=reduced intensity conditioning.

As we observed that both the recipient and donor haplotype showed association with aGvHD, we elucidated how their combinations influenced the risk. We grouped patients into four categories based on the recipient and donor haplotypes; in the first group neither

the recipient nor the donor was a 46/1 haplotype carrier (R0D0, n=43), in the second group only the donor (R0D1, n=21), whereas in the third only the recipient was a 46/1 haplotype carrier (R1D0, n=23), and haplotype carrier patients with a carrier donor belonged to the final group (R1D1, n=37). In the four groups, aGvHD grades II-IV incidence rates were the following; 13.9% in the R0D0, 28.6% in the R0D1, 34.8% in the R1D0, and 45.9% in the R1D1 group (**Figure 10.**). The analysis confirmed significantly higher risk if both, the recipient and the donor (R1D1) were 46/1 haplotype carriers in comparison with patients with R0D0 constellation (OR=5.242, 95% CI=1.784-15.404, p=0.003).

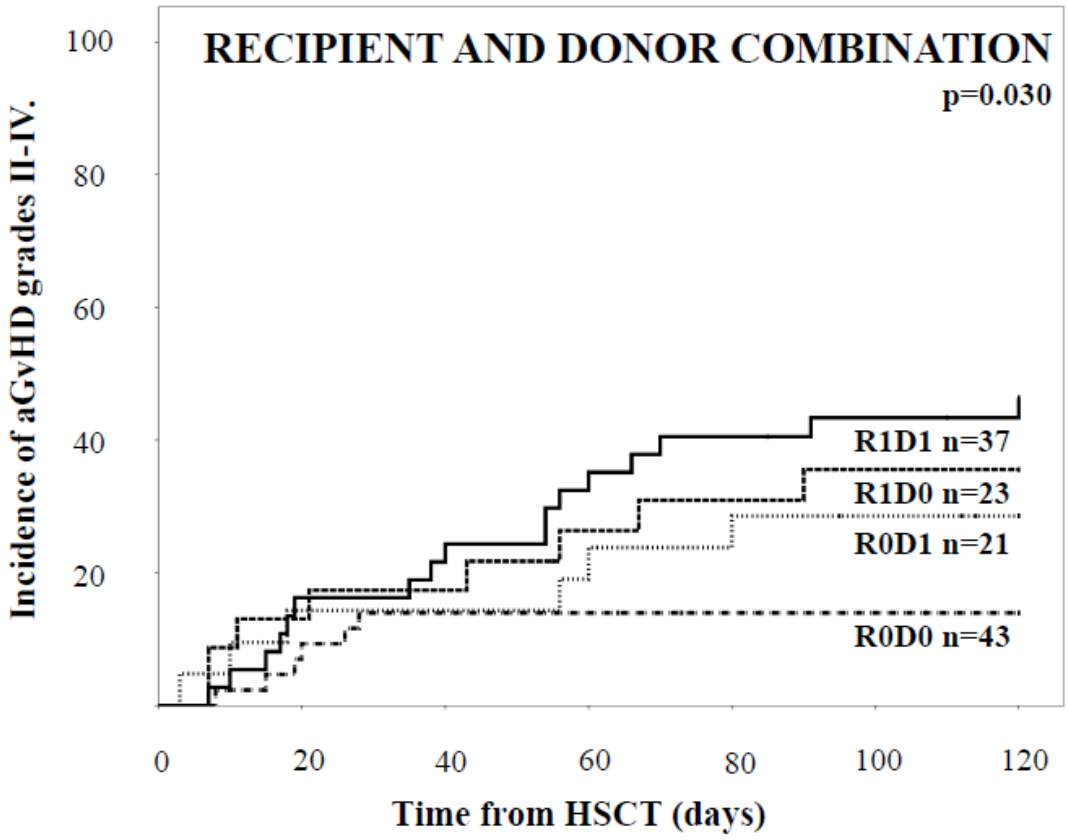

Figure 10. Incidence of aGvHD grades II-IV according to recipient/donor haplotype pairs applying a dominant model. Incidences were 13.9% in the R0D0, 28.6% in the R0D1, 34.8% in the R1D0, and 45.9% in the R1D1 group (p=0.030).

Abbreviations for Figure 10. aGvHD=acute graft-versus-host disease, HSCT= haematopoietic stem cell transplantation, P=probability value, R0D0=non-carrier recipient and donor, R0D1=non-carrier recipient and carrier donor, R1D0=carrier recipient and non-carrier donor, R1D1=carrier recipient and donor.

The relapse rate showed variation in the four groups occurring with the highest frequency in the R0D1 subgroup (7/21, 33.3%), followed by the R0D0 group (8/43, 18.6%), but it was low in the two remaining groups [2/23, 8.7% in the R1D0 (p=0.064 vs. R0D1) and 1/37, 2.7% in the R1D1 group (p=0.0024 vs. R0D1)]. Death due to transplantation related complications accounted for 46.2% (6/13) in the R0D0, 60.0% (3/5) in the R1D0, 60.0% (6/10) in the R0D1 and 93.8 % (15/16) in the R1D1 subgroup (p=0.0097 vs. R0D0). The 2-year OS rates were comparable in the four above described recipient-donor groups [69.0±7.6% (R0D0), 82.1±8.1% (R1D0), 42.1±13.3% (R0D1) and 61.1±8.2% (R1D1), p=0.211].

6. DISCUSSION

6.1. TA-TMA study

To our knowledge, our group has been the first investigating the association of TA-TMA with HLA-DRB1*11 carriership besides validating multiple other previously published risk factors in a large single centre allo-HSCT cohort. As a new finding, our observations suggest that HLA-DRB1*11 may predispose for TA-TMA development and impact on the survival of recipients affected by TA-TMA.

Definitions for TA-TMA in literature vary significantly (140). Reported data show huge variations with regards to disease incidence (0-75%), risk factors and mortality (median 75%), due partly to lack of consensus diagnostic criteria (26, 227, 141, 152). This issue was highlighted in a review article, which found 28 different definitions in 35 reports for the diagnosis of TA-TMA (26). Therefore international efforts were made to harmonise the diagnostic algorithms and the following three diagnostic criteria were suggested: Blood and Marrow Transplant Clinical Trials Network (BMT CTN, 2005) (151), International Working Group (2007), (155) and overall TMA including probable TMA (2010) (156). Soon following the publication of the recommendations their limitations were also explored (156, 228, 143). In our work patients were categorised according to two of the above criteria (156, 151). In 2014, an international panel suggested updated diagnostic guidelines (140, 153). In our study, the adaptation of the proposed criteria was hindered by lack of serum complement C5b-9 measurements, which is not part of routine follow-up in post-transplant patients. Furthermore this recommendation was published in 2014, whereas our study incorporated patients treated between 2007 and 2013, and data collection and analyses for this study preceded the latter proposal.

In our cohort, TA-TMA was diagnosed in 18.8% with a predominant occurrence in the early post-transplant period in keeping with previous publications (140). In line with earlier reports several risk factors for the development of TA-TMA were established, such as unrelated donor type, aGvHD grades II-IV, myeloablative conditioning (MAC), high-dose busulfan in the MAC regimen, tacrolimus (TAC)-based immunosuppression, CMV reactivation/disease and, as a novel finding carriership for HLA-DRB1*11. In good

agreement with the literature, one of the strongest predictors for TA-TMA was aGvHD grades II-IV (174, 175, 141, 162, 159, 160). The recent recognition of a new cytokine axis (suppression of tumorigenicity 2 [ST2]/IL-33) has recently delineated the possible link between GvHD and TA-TMA (229). Vander Lugt et al. confirmed the predictive value of high ST2 levels at day 14 for higher NRM and the association of increased levels at diagnosis of aGvHD with therapy refractoriness (124). ST2 is a marker of endothelial injury, a common feature in aGvHD and TA-TMA, and in a publication from March 2017 elevated ST2 level was significantly associated with TA-TMA (229). The authors speculated that their finding might possibly suggest that TA-TMA could have contributed to the excess mortality observed by Vander Lugt et al. and that a proportion of patients with TA-TMA might have been misclassified as therapy-resistant GvHD (229). The ST2/IL-33 axis has become the focus of extensive research and in animal studies blockade of IL-33 and ST2 interactions efficiently reduced the lethality of GvHD (125). Preliminary results are promising suggesting that the axis could be a novel target for GvHD therapy and considering the link with TA-TMA perhaps also for TA-TMA (125).

It has been clearly documented that calcineurin inhibitors and mammalian target of rapamycin inhibitors administered routinely for GvHD prophylaxis or treatment in the allo-HSCT setting can induce TA-TMA (142, 26, 143). Several publications have suggested that the combination of TAC and sirolimus (SIR) substantially increases the risk of TA-TMA (230, 170, 171). In our cohort, the majority of patients were treated with TAC/SIR combination and we observed a significantly higher TA-TMA incidence with TAC-based medications in comparison with CSA-based protocols. However, TA-TMA was not more common with TAC and SIR combinations compared to other TAC-containing regimens, in line with findings of Labrador et al. (162). According to our institutional protocol, SIR was routinely terminated early, 30 days post-transplant if GvHD had not developed within the first 30 days, which might have contributed to the above observation.

With regard to conditioning intensity, controversial results have been described and some studies showed a differential risk for RIC and MAC, while others failed to confirm an association (142, 141, 162, 166, 160). In this study, we detected an increased risk of TA-TMA with MAC. However, our observations might be biased, because predominantly

TAC-based immunosuppression was administered in the MAC-treated subgroup (93% vs. 48% in RIC), and therefore myeloablative conditioning and TAC together could have had an amplified impact. We also validated previous observations on substantially higher risk of TA-TMA with high-dose busulfan-containing conditioning regimens (142, 141, 170). The contribution of different fungal and viral infections to TA-TMA appearance has also been indicated (163, 162, 143). Similarly to earlier reports, we found an association of TA-TMA with CMV reactivation/disease (167). Unlike some data, we could not identify significant associations of female gender, lymphoid malignancies or ABO incompatibility with TA-TMA (142, 162, 159, 231, 173).

As a novel finding, our data indicates that HLA-DRB1*11 may also play a role in the development of TA-TMA. Together with previous reports, the findings suggest that, HLA-DRB1*11 is associated with syndromes in the spectrum of thrombotic microangiopathies, such as TTP, HUS and TA-TMA (232, 147-150). The observed association of HLA-DRB1*11 with TA-TMA supports the theory that the role of HLA-DRB1*11 and/or closely linked genes in the pathogenesis of thrombotic microangiopathies might be complex and extend beyond susceptibility to autoantibody formation against ADAMTS13, which is not the hallmark of TA-TMA. Literature data is extensive concerning the association of HLA-DRB1*11 with certain autoimmune, infectious and malignant conditions. Overrepresentation was described in systemic sclerosis, early-onset juvenile chronic arthritis, lichen planopilaris, Henoch-Schönlein purpura, Helicobacter pylori positive idiopathic thrombocytopenic purpura, hairy cell leukaemia, familial chronic lymphocytic leukaemia, cervical cancer and Kaposi sarcoma (232-241). Interestingly, in hairy cell leukaemia, which itself was enriched in HLA-DRB1*11 carriers, a complication of anti-CD22 recombinant immunotoxin BL22 treatment, HUS, (another, not ADAMTS13 deficiency-associated form of TMA) was also associated with HLA-DRB1*11 (232). Decreased HLA-DRB1*11 frequency was observed in chronic hepatitis C infection, hepatocellular carcinoma and rheumatic heart disease (242-244). HLA-DRB1*11 was also linked to drug-induced immune-mediated conditions (e.g. myositis in HLA-DRB1*11 carriers exposed to statins) (245). The wide clinical spectrum of HLA-DRB1*11-associated conditions advocates for the co-occurrence of additional provoking factors, because a unique mechanism is unlikely to drive all the above mentioned conditions.

We speculate that in the presence of a predisposing genetic risk, such as HLA-DRB1*11 less robust acquired components, e.g. calcineurin inhibitors could induce the manifestation of TA-TMA. In patients with no such genetic background, more aggressive risk factors like aGvHD might be needed to provoke endothelial dysfunction and damage. In view of the association of HLA-DRB1*11 with TA-TMA, in carriers caution with other proven risk factors e.g. administration of TAC and SIR or high-dose busulfan-containing conditioning might be of clinical benefit.

We observed a higher proportion of HLA-DRB1*11 carriers in the unrelated setting compared to the sibling subgroup and to that of the data of the Hungarian Marrow Registry. This might be explained by the fact that HLA-matched donor selection could have been more successful for HLA-DRB1*11 recipients giving that HLA-DRB1*11 was the most frequent HLA-DRB1 allele group in the cohort. To overcome the above bias, we performed subgroup analyses for related and unrelated donor transplants separately, which confirmed a significant association of HLA-DRB1*11 with TA-TMA only in the sibling subgroup ($p=0.047$), but not in the unrelated donor subgroup. The explanation for the finding might be that in unrelated donor transplantation, the role of a genetic factor, such as HLA-DRB1*11 might be overcome by other more powerful risk factors, such as genetic disparity between recipient and donor and higher incidence of moderate to severe aGvHD.

We clearly determined the significant adverse effect of TA-TMA on survival in keeping with previous findings (174, 156, 153). Moreover the analyses showed that the presence of organ damage further worsened the prognosis among TA-TMA patients. Our findings support the recommendation by Cho et al. proposing that if only patients with organ damage were considered for the diagnosis of TA-TMA, cases would be missed and underdiagnosed. However even those cases with attributes suggestive of TA-TMA without organ involvement have a worse prognosis (156).

With regards to survival data our analyses showed that in patients suffering from TA-TMA, the survival of HLA-DRB1*11 carriers was better compared to non-carriers. In the whole cohort though the survival did not differ according to HLA-DRB1*11. Among HLA-DRB1*11 carrier patients with TA-TMA without organ involvement the prognosis

was excellent. The survival difference in our cohort cannot be explained by the uneven distribution of HLA-DRB1*11 according to donor type, considering the generally inferior outcome of unrelated transplantations, which were overrepresented in HLA-DRB1*11-positive cases. The presence or absence of organ involvement cannot justify our observation either given that the development of organ damage was comparable in HLA-DRB1*11-positive and HLA-DRB1*11-negative TA-TMA cases. The strong association of TTP with HLA-DRB1*11 was clearly documented in a number of studies, and considering the extremely poor prognosis of TTP without total plasma exchange (TPE), it is difficult to interpret why in our TA-TMA cohort HLA-DRB1*11 carriership granted a more favourable survival, although patients were not treated with TPE. The above emphasises that HLA-DRB1*11 might induce thrombotic microangiopathy not via facilitating autoantibody formation against ADAMTS13, but via other routes. Whether the risk might be affected by other genes inherited in strong linkage disequilibrium with HLA-DRB1*11 alleles is to be investigated. We hypothesize that carrying HLA-DRB1*11 might constitute a genetic predisposition for the initiation of the pathological process leading to the endothelial injury essential for TA-TMA development, but with the diagnosis made early and relevant measures taken, recovery can be achieved easier compared to cases with no such genetic background. Although general data does not support TPE in TA-TMA, it is hypothetically possible that the subpopulation of HLA-DRB1*11 positive patients with TA-TMA could respond better to this modality. The exact mechanism how HLA-DRB1*11 contributes to TA-TMA development is yet to be explored.

In summary, the observations of our study suggest that carriership of HLA-DRB1*11 predisposes for TA-TMA and among those affected by TA-TMA HLA-DRB1*11 favourably influences the survival compared with non-carriers. The exact pathomechanism remains to be identified. However, due to the limitations of the retrospective nature of the study, the role of HLA-DRB1*11 in TA-TMA should be addressed by other groups, preferentially in a prospective design. Investigation could look into whether response to treatment modalities such as TPE or eculizumab differs in HLA-DRB1*11 carriers and non-carriers.

6.2. JAK2 46/1 haplotype study

Given the essential role of JAK/STAT pathway in graft-versus-host reactions and the documented association of JAK2 46/1 haplotype with inflammation we hypothesised that this inherited variant could influence aGvHD risk. The proposition was approved by this work and as a novel finding we confirmed the association of the recipient and the donor JAK2 46/1 haplotypes with aGvHD grades II-IV in AML patients undergoing allo-HSCT.

To date hundreds of thousands of patients have benefitted from allo-HSCT, which have delivered cure to malignant, metabolic and immunological conditions. Notwithstanding aGvHD remains the major impediment to its broader application accounting for significant morbidity and mortality (24, 246). Acute GvHD develops following the recognition of disparate host-specific proteins by donor T cells (24). Unequivocally mismatching of the major histocompatibility antigens are of utmost importance for the initiation of graft-versus-host responses (247, 84). Nonetheless, aGvHD arises in up to 45% of recipients of fully HLA-matched sibling grafts, pointing out the contribution of non-HLA genes and proteins to the alloimmune reactions (97, 24). In our patient cohort 95.2% of patients received HLA-A, -B and -DR antigen matched grafts, and 88.7 % were transplanted from 8/8 matched donors (considering HLA-A, -B, -C and DRB1 antigens), making our patient population ideal for studying the role of non-HLA genes.

To date, hundreds of non-HLA germline variants have been tested in relation to HSCT outcomes (93, 95). Polymorphisms of genes involved in cytokine-mediated pathways have been the most extensively studied. Several single nucleotide polymorphisms have showed association with the occurrence and severity of acute and/or chronic GvHD, relapse rate and survival [in genes of the following cytokines and/or their receptors; IFNG, IL-1, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17, IL-18, IL-23, TGFB1 and TNFA] (115, 93, 248, 249, 97, 98, 94, 95, 250). Additionally, biomarker studies are also supportive of the role of cytokines in the alloimmune reactions implicating correlations between serum levels of cytokines, their receptors and GvHD manifestation, severity and survival (115, 121, 251-253, 124).

JAK2 is a ubiquitously expressed tyrosine kinase member of the JAK/STAT family (191). The pathway is activated via binding of multiple cytokines,

controlling inflammation, immunity and haematopoiesis (192). Aberrant activation of the JAK/STAT pathway has been proven in several haematological and solid cancers, as well as in immune-mediated conditions, including GvHD (31, 192-194). JAK2 relays the signals of many cytokines with relevance for GvHD (such as IL-6, IL-12, IL-23, IFNG), therefore our hypothesis was that polymorphisms of JAK2 might affect the manifestation of GvHD (192, 191). To our knowledge prior to our study the role of JAK2 polymorphisms had not been tested in the context of aGvHD. Among JAK2 variants, the investigation of the 46/1 haplotype seemed to be relevant, as it was reported to have functional consequences (203, 202). Although 46/1 haplotype consists of several closely linked polymorphisms, previous studies identified tag SNPs (e.g. rs12340895, rs12343867 or rs10974944) in complete linkage disequilibrium with the haplotype. Screening for a single tag SNP is a widely accepted method for 46/1 haplotype determinations and in our study we selected rs12343867 for the identification of the haplotype. (209, 210)

Our study assessed both recipient and donor haplotypes, as the release and signalling of cytokines might be dependent on both genotypes. The classical stepwise pathophysiological model of aGvHD described the inflammatory course as a three-step process (24). The first step includes tissue damage in the host induced by preparative regimens resulting in the release of pro-inflammatory cytokines. This is followed by the activation of recipient and donor-derived antigen presenting cells (APCs) and donor T cell activation in the second phase. In the third, effector phase the expansion of activated donor T cells leads to T cell-mediated cytotoxicity against the host (24). Cytokines significantly amplify aGvHD responses, which explains why polymorphisms of cytokine genes could influence the risk of the complication (24). The activation of cytokine pathways occurs prior to engraftment and remains crucial throughout, explaining why recipient-derived polymorphisms could be at least as critical for aGvHD presentation as those of donor origin (115, 120). Our observation, that both, recipient and donor JAK2 haplotype influenced the development of moderate to severe aGvHD can be supported by the above described pathomechanism. Underlining the independent role of both haplotypes, the highest aGvHD grades II-IV incidence was found among those patients, who themselves and their donors also carried the haplotype (R1D1: 45.9%), whereas a

single 46/1 haplotype carriership either of the recipient or the donor resulted in an intermediate rise in aGvHD (R1D0: 34.8% and R0D1: 28.6%) compared to double negative cases (R0D0: 13.9%). The role of the recipient 46/1 haplotype seemed to be more relevant in our cohort as its association with aGvHD remained independent in multivariate analyses. Other cytokine polymorphism studies also revealed that the total number of the SNPs in the recipient/donor pair influenced the risk of complications e.g. the presence of 3-4 SNPs vs. 0-2 SNP at codon 10 of transforming growth factor beta 1 (TGFB1) was associated with inferior outcome (111). Interestingly in this study similarly to our observations the recipient genotype significantly influenced the outcome, but the donor on its own not (111). In view of previous reports on the association of JAK2 46/1 haplotype with inflammatory bowel disease (IBD), and that IBD and aGvHD share common immunological attributes, we separately assessed the occurrence of gastrointestinal aGvHD (218, 254). Both patient and donor haplotype influenced gastrointestinal manifestation of the disease.

The recipient haplotype also affected relapse rate, inversely to that of aGvHD risk, suggesting that the recipient haplotype might be involved in both graft-versus-host and graft-versus-leukaemia reactions. Overall survival depending on the recipient carrier status did not differ probably due to the counteracting effect of increased aGvHD and reduced relapse rates. Similar association was not observed between relapse and donor haplotype.

Further supportive evidence for the critical role of the JAK2 signalling in the development of aGvHD comes from the successful application of JAK1/JAK2 inhibitors in the management of GvHD. Experiments showed that blockade of JAK1/JAK2 diminished alloreactive T cell trafficking into GvHD affected organs, reduced GvHD score and improved survival (197, 199, 200, 198). In a mouse model ruxolitinib, a JAK1/2 inhibitor resulted in the reduction of pro-inflammatory cytokine production, impaired the differentiation of CD4+ effector T cells, and promoted regulatory T cells. As a consequence, JAK1/2 inhibition reduced aGvHD even in a major mismatch model (201). The same group went on to investigate whether the findings of the animal models could be translated into clinical practice, and successfully treated six patients with steroid-refractory acute or chronic GvHD with low-dose ruxolitinib (201). In a most recent

multicentre study the overall response rate to ruxolitinib for severe (grades III-IV), steroid-refractory aGvHD was exceptionally high, 81.5 % (44/54) with 46.3% CR rate (139). Further trials are warranted, but the initial results are very promising, indicating that the blockade of JAK1/2 could be a rational approach for the treatment and prevention of GvHD. Future studies could address the effectiveness of JAK2 inhibitors in JAK2 46/1 haplotype carriers and in non-carriers separately.

Although the exact pathomechanism how the JAK2 46/1 haplotype alters JAK2 function and predisposes to aGvHD is yet to be revealed, assumptions from MPN and IBD studies might be translated into the context of aGvHD. Earlier hypotheses suggested that JAK2 46/1 haplotype might be a marker for altered myelomonocytic response to cytokines, leading to increased risk of inflammation, infections and myeloid neoplasms (203, 202).

We acknowledge the two major limitations of our study, the limited sample size and the lack of an independent confirmatory cohort. Further validation studies are warranted, but our preliminary findings are supportive of the role of polymorphisms in the JAK/STAT pathway in alloimmunity.

7. CONCLUSIONS

1. Our work identified two novel risk factors for the development of serious post-transplant complications, transplantation-associated thrombotic microangiopathy and acute graft-versus-host disease in this single centre cohort of adult Hungarian patients undergoing allogeneic HSCT.

2. TA-TMA was diagnosed in nearly the fifth of the patients with predominant presentation in the early post-transplant period (median 40 days, in 81% within first 100 days of transplantation). Unrelated donor type, acute GvHD grades II-IV, myeloablative conditioning, tacrolimus-based immunosuppression, CMV reactivation/disease and as a novel finding carriership for HLA-DRB1*11 were identified as risk factors for TA-TMA development.

3. In multivariate analyses TA-TMA was an independent adverse risk factor for survival. Within the TA-TMA group the presence of organ damage adversely affected the outcome, whereas the outcome was more favourable for HLA-DRB1*11 carriers.

4. In the AML cohort we confirmed as a new observation that both, recipient and donor JAK2 46/1 haplotypes were associated with the development of aGvHD grades II-IV. Moreover, the impact of the recipient and donor genetic variant seemed to be cumulative giving that the highest incidence was disclosed among those, who themselves and their donors were both haplotype carriers. Multivariate analyses confirmed that the recipient JAK2 46/1 haplotype was independently related to aGvHD, however the donor JAK2 46/1 haplotype was not.

5. Recipient JAK2 46/1 haplotype showed a favourable correlation with relapse rate, but the donor JAK2 46/1 haplotype had no effect on relapse incidence.

6. The recipient and donor JAK2 46/1 haplotypes had no significant impact on overall survival.

8. SUMMARY

Allogeneic haematopoietic stem cell transplantation (allo-HSCT) is the only curative treatment for many conditions. The procedure can be followed by severe, life-threatening complications, such as acute graft-versus-host disease (aGvHD) or transplantation-associated thrombotic microangiopathy (TA-TMA). In our work we assessed the association of the two above conditions with genetic factors. A major achievement of this multidisciplinary collaborative study was the setting up of a clinical database including data of all adult patients (n=425) who underwent first allo-HSCT in Hungary between 2007 and 2013 for malignant disease indications. Our first study assessed the incidence, clinical features and risk factors of TA-TMA, with special view to the hypothesised role of HLA-DRB1*11 in its development. We confirmed the role of several previously established risk factors for TA-TMA development, such as unrelated donor type, aGvHD grades II-IV, myeloablative conditioning, tacrolimus-based immunosuppression and CMV reactivation/disease, among which the first two were independently associated in multivariate analyses. As a novel finding we demonstrated that carriership of HLA-DRB1*11 also predisposed for TA-TMA. We clearly determined a strong negative impact of TA-TMA on survival with even more dismal prognosis in TA-TMA patients with organ damage. Among patients affected by TA-TMA, HLA-DRB1*11 favourably influenced survival. In the AML subgroup of the total cohort we conducted a second study and investigated the association of recipient and donor JAK2 46/1 haplotypes with grades II-IV aGvHD, the condition that remains the major impediment of allo-HSCT due to significant morbidity and mortality. We confirmed that both recipient and donor JAK2 46/1 haplotypes were significantly associated with grades II-IV aGvHD, and their impact seemed to be additive as the risk was highest among those haplotype carrier recipients whose donors were also haplotype carriers. In multivariate analyses recipient haplotype showed independent association with aGvHD. The incidence of relapse was lower among haplotype carriers, but overall survival did not differ significantly according to recipient or donor haplotype.

9. ÖSSZEFOGLALÁS

Az allogén hemopoetikus őssejt transzplantáció (allo-HSCT) számos megbetegedés esetén az egyetlen, tartós gyógyulást biztosító terápia. A beavatkozást súlyos, magas mortalitással járó szövődmények kísérhetik, mint az akut graft-versus-host betegség (aGvHD) vagy a transzplantációhoz társuló trombotikus microangiopathia (TA-TMA). Munkánk során a fenti két komplikáció genetikai faktorokkal való összefüggését vizsgáltuk. Munkánk egyik legfőbb eredményének tekintjük, hogy társszakmák szoros közreműködésével létrehoztunk egy klinikai adatbázist azon 425 felnőtt beteg adataival, akik Magyarországon 2007 és 2013 között első allo-HSCT-ben részesültek malignus alapbetegség miatt. A klinikai adatok felhasználásával született első munkánkban a TA-TMA előfordulási gyakoriságát, klinikai jellemzőit és rizikófaktorait vizsgáltuk, különös tekintettel a HLA-DRB1*11 gén feltételezett szerepére. Megerősítettük számos, korábban leírt rizikófaktor szerepét, mint az idegen donoros átültetés, II-IV. súlyossági fokú aGvHD, mieloablatív kondicionálás, tacrolimus-alapú GvHD prevenció és CMV reaktiváció/betegség, melyek közül az első kettő szerepe függetlennek bizonyult multivariáns analízis során. Új eredményként igazoltuk a HLA-DRB1*11 hordozás hajlamosító szerepét. Kimutattuk a TA-TMA túlélésre kifejtett kedvezőtlen hatását, mely még kifejezettebb volt a célszerv károsodást elszenvedett betegek között. TA-TMA-ban szenvedő betegek körében a HLA-DRB1*11 hordozás kedvezően befolyásolta a túlélést. A teljes csoport AML alcsoportjában került sor második vizsgálatunkra, melyben a recipiens és donor JAK2 46/1 haplotípus szerepét vizsgáltuk II-IV. fokozatú aGvHD kialakulására, mely szövődmény képezi mindmáig az allogén transzplantáció egyik legfőbb akadályát a következményes magas morbiditás és mortalitás miatt. Vizsgálatunkban összefüggést találtunk a recipiens és donor JAK2 46/1 haplotípus és az aGvHD között. A recipiens és donor haplotípus szerepe additívnak bizonyult, mivel a legnagyobb kockázatot abban a csoportban észleltük, amelyben mind a recipiens, mind a donor hordozók voltak. A recipiens haplotípus szerepe független volt multivariáns analízis során. A haplotípust hordozó betegek körében kevesebb volt a relapsus, de a teljes túlélés nem különbözött szignifikánsan a recipiens vagy donor haplotípus függvényében.

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11. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

11.1. Candidate's publications related to the theme of the thesis

1. **Balassa K**, Krahling T, Remenyi P, Batai A, Bors A, Kiss KP, Torbagyi E, Gopcsa L, Lengyel L, Barta A, Varga G, Tordai A, Masszi T, Andrikovics H. (2017) Recipient and donor JAK2 46/1 haplotypes are associated with acute graft-versus-host disease following allogeneic hematopoietic stem cell transplantation. Leuk Lymphoma, 58: 391-398. **IF: 2.755 (2016)**

2. **Balassa K**, Andrikovics H, Remenyi P, Batai A, Bors A, Kiss KP, Szilvasi A, Rajczy K, Inotai D, Gopcsa L, Lengyel L, Barta A, Reti M, Tordai A, Masszi T. (2015) The potential role of HLA-DRB1*11 in the development and outcome of haematopoietic stem cell transplantation-associated thrombotic microangiopathy. Bone Marrow Transplant, 50: 1321-1325. **IF: 3.636**

11.2. Candidate's publications not related to the theme of the thesis

1. Batai A, Remenyi P, Reti M, Barta A, Gopcsa L, Lengyel L, Torbagyi E, Csukly Z, Karaszi E, Tordai A, Andrikovics H, **Balassa K**, Tasnady S, Masszi T. (2017) Allogeneic hematopoietic stem cell transplantation in Hungary. Orv Hetil, 158: 291-297.

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