Research Paper

Intestinal Adenovirus Shedding Before Allogeneic Stem Cell Transplantation Is a Risk Factor for Invasive Infection Post-transplant

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ABSTRACT

Human adenoviruses (HAdV) are a major cause of morbidity and mortality in pediatric human stem cell transplant (HSCT) recipients. Our previous studies identified the gastrointestinal tract as a site of HAdV persistence, but the role of intestinal virus shedding pre-transplant for the risk of ensuing invasive infection has not been entirely elucidated. Molecular HAdV monitoring of serial stool samples using RQ-PCR was performed in 304 children undergoing allogeneic HSCT. Analysis of stool and peripheral blood specimens was performed pre-transplant and at short intervals until day 100 post-HSCT. The virus was detected in the stool of 129 patients (42%), and 42 tested positive already before HSCT. The patients displaying HAdV shedding pre-transplant showed a significantly earlier increase of intestinal HAdV levels above the critical threshold associated with high risk of invasive infection ($p < 0.01$). In this subset of patients, the occurrence of invasive infection characterized by viremia was significantly higher than in patients without HAdV shedding before HSCT (33% vs 7%; $p < 0.0001$). The data demonstrate that intestinal HAdV shedding before HSCT confers a greatly increased risk for invasive infection and disseminated disease post-transplant, and highlights the need for timely HAdV monitoring and pre-emptive therapeutic considerations in HSCT recipients.

1. Introduction

Human adenovirus (HAdV) infections are associated with life-threatening complications in immunocompromised individuals including particularly children undergoing human allogeneic stem cell transplantation (HSCT) (Feghoul et al., 2015; Lion et al., 2010; Mynarek et al., 2014; van Tol et al., 2005). In this setting, viral reactivation is reportedly more prevalent than de-novo infection (Veltrop-Duits et al., 2011). Primary infections in immunocompetent individuals occur most commonly in early childhood, often without any clinical symptoms, but HAdV can subsequently persist in tonsillar and adenoidal lymphocytes as well as in the gastrointestinal (GI) tract (Roy et al., 2011; Garnett et al., 2002). The role of the GI-tract as a sanctuary for HAdV persistence has been discussed for decades, because shedding of the virus into stool was found in immunocompetent individuals even without any evidence of disease (Adrian et al., 1988). In HSCT recipients, increasing HAdV loads in serial stool specimens have been documented during the post-transplant period prior to invasive infection, supporting the notion of intestinal virus persistence and reactivation (Feghoul et al., 2015; Lion et al., 2010; Jeulin et al., 2011). Our recent study in immunocompetent pediatric patients revealed HAdV persistence in mucosal lymphoid cells in the GI-tract, with particularly high prevalence in the ileum, whereas in HSCT recipients with HAdV reactivation post-transplant, an enormous virus density has been documented in intestinal epithelial cells (Kosulin et al., 2016a). These observations revealed that intestinal lymphocytes apparently serve as a major site of HAdV persistence, but epithelial cells in the GI-tract are exploited for massive virus production (Kosulin et al., 2016a). The highly productive virus replication in epithelial cells explains the rapidly expanding virus numbers in stool specimens of HSCT recipients prior to the onset of viremia and disseminated disease (Feghoul et al., 2015; Lion et al., 2010; Mynarek et al., 2014; Berciaud et al., 2012).

According to the current ECIL (European Conference of Infections in Leukemia) guidelines, peripheral blood (PB) samples are recommended as the primary material for HAdV screening in HSCT recipients (Matthes-Martin et al., 2012). There is, however, increasing evidence reported by different centers that rapidly increasing HAdV DNA copy numbers exceeding defined threshold levels in serial stool samples herald invasive infection days to weeks before the virus becomes

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detectable in PB (Feghoul et al., 2015; Lion et al., 2010; Mynarek et al., 2014; Jeulin et al., 2011). The monitoring of HAdV DNA copy numbers in stool could therefore serve as a basis for timely initiation of pre-emptive therapy, in attempts to prevent invasive infection. Disseminated HAdV-mediated disease is associated with dismal outcome, partly because delayed onset of therapy by current treatment options often fails to control adenovirus-related complications (Feuchtinger et al., 2006; Lion, 2014). Since HAdV persistence in the GI-tract of immunocompetent children and post-transplant reactivation of the virus in pediatric patients were shown to occur at similar frequencies (Kosulin et al., 2016a), we hypothesized that detection of HAdV shedding into stool before HSCT may provide an early marker for the risk of viremia and HAdV-related disease post-transplant. To address this notion, the present study involving a large cohort of pediatric HSCT recipients was performed.

2. Material and Methods

2.1. Patients

The samples studied included serial stool and peripheral blood specimens from 304 consecutive patients (Table 1) who underwent allogeneic HSCT at the St. Anna Children's Hospital, Vienna, Austria, between 2000 and 2015. The samples were collected as part of the routine HAdV screening starting before conditioning, and subsequently at seven-day intervals until day +100 post-transplant. Only patients with available stool samples before HSCT were included. Since the present study was based exclusively on the analysis of results obtained by routine diagnostic testing, for which written informed consent had been obtained from each patient and/or the parents, additional approval by the ethics committee was not required to ensure that the study is carried out in agreement with the Helsinki-Declaration. Patients with HAdV viremia received antiviral therapy with cidofovir, sometimes in combination with ribavirin, in the presence of HAdV species C. In

Table 1
Patient and transplant characteristics (n = 304).

Median age (years)	9.3 (range:0.2–28.0)	%
Underlying disease		
Acute leukemia	134	44
Chronic myeloid leukemia	23	8
Myelodysplastic syndrome	23	8
Lymphoma	19	6
Solid tumor	19	6
Severe aplastic anemia	13	4
Hemoglobinopathy	14	4.6
Fanconi's anemia	9	3
Immunodeficiency	41	13.5
Metabolic disorders	8	2.6
Idiopathic hypereosinophilia	1	0.3
Donor		
Matched sibling donor	92	30
Matched family donor	3	1
Mismatched family donor	35	12
Unrelated donor	174	57
Graft		
Bone marrow	219	72
Unmanipulated peripheral stem cells cells SC	63	20.7
T cell-depleted peripheral stem cells	17	5.6
Cord blood	5	1.7
GvHD		
aGvHD ≥ grade 2	63	21
aGvHD < grade 2	241	79
Lymphocyte reconstitution		
CD3$^+$ > 300 cells/μl	175	58
CD3$^+$ < 300 cells/μl	129	42

cases of failed response to antivirals and absence of HAdV-specific T-cells, adoptive T-cell transfer with donor-derived HAdV-specific T cells was offered (Lion et al., 2010; Lion, 2014).

2.2. Isolation of Viral DNA

The QIAamp DNA Mini Kit (Qiagen) was employed for isolation of DNA from peripheral blood (PB). Extraction of DNA from stool specimens was performed by the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's recommendations.

2.3. RQ-PCR Analysis

A broad-spectrum real-time quantitative (RQ)-PCR assay including 50 cycles of amplification was employed for HAdV-screening using the ABI 7500 Sequence Detectors (Thermo Fisher Scientific, Waltham, MA, USA), as described previously (Ebner et al., 2005; Kosulin et al., 2016b). Specimens that tested positive for HAdV by the broad-spectrum RQ-PCR screening assay were analyzed by PCR tests specifically identifying individual HAdV species (Lion et al., 2003). The detection limit of these tests is 10 virus DNA copies per PCR reaction, and specimens displaying ≥ 5x10E2 DNA copies/g stool by the assay employed were regarded as positive. Similarly, adenoviremia was defined as HAdV detectable in peripheral blood at levels ≥ 5x10E2 DNA copies/ml. In contrast to blood specimens where the virus concentration is indicated as copy number per ml blood, in stool it is denoted as virus copy number per gram of the sample because the presence of high levels of free lytic viruses cannot be excluded.

2.4. Statistics

Fisher's exact test or exact chi-square test were used to analyze the correlation between adenoviremia until day 100 after HSCT and HAdV positivity in stool before and after HSCT, considering other treatment- and patient-related characteristics including donor type, graft source, graft-versus-host disease (GvHD) of all grades, and T-cell reconstitution. Multivariate analysis was done using logistic regression with Firth correction. Odds ratios (OR) with the respective 95% confidence intervals (CI) are indicated in addition to p-values in univariate and multivariate analysis. Odds ratios are not defined in the absence of adenoviremia in one group. In this instance, only the lower limit of the 95% CI for the OR is indicated. In 262 of 304 patients, a single transplantation was performed, while two or three consecutive transplantations were done in 37 of 304 and 8 of 304 patients, respectively. The variables were assessed considering the first transplantations only (Table 2). All indicated p-values are two sided, and values ≤ 0.05 were considered significant.

3. Results

3.1. Onset of HAdV Shedding Into Stool and Post-transplant Kinetics of Intestinal Virus Levels

Pan-adenoviral RQ-PCR screening of serial stool specimens revealed positive results in 129 of 304 (42%) pediatric HSCT patients analyzed. Intestinal shedding of the virus was detected already prior to transplantation in 42 individuals, corresponding to 14% of the entire cohort (n = 304), while 87 patients revealed first HAdV positive test results in stool only after transplantation. In the majority of patients within the latter subset (n = 74), HAdV was detected within 56 days post-transplant, including 32 patients with first virus detection within 14 days, 23 between 15 and 28 days, 10 between 29 and 42 days, and 9 between 43 and 56 days after transplantation, respectively (Fig. 1A). Only 13 patients showed first HAdV positivity in stool beyond day 56 post-transplant.

Table 2
Univariate and multivariate analysis of risk factors for viremia post-transplant.

Parameter (univariate)		Viremia	p-Value
HAdV in stool			
Negative		0%	<0.0001
Positive after HSCT		33%	
Positive before HSCT		22%	
Donor type			
MSD/MFD		2%	0.004
MMFD		18%	
UD		14%	
Source			
BM		7%	0.001
pPSC		21%	
CB + combined		17%	
aGVHD			
No		12%	0.403
Yes		9%	
Lymphocyte reconstitution			
CD3$^+$ < 300 cells/μl		13%	0.432
CD3$^+$ > 300 cells/μl		10%	

Parameter (multivariate)	Effect coding[a] p-value	Reference coding[b] p-value	OR (95% CI)
HAdV in stool			
Positivity after HSCT vs negativity	0.019	0.0009	>6[c]
Positivity before HSCT vs negativity	<0.0001	P < 0.0001	>14.3[c]
Donor type			
MMFD vs UD	0.025	0.011	0.13 (0.03–0.63)
MFD, MSD vs UD	0.37	0.23	0.72 (0.19–2.8)
Lymphocyte reconstitution			
CD3$^+$ < 300 cells/μl	0.25	0.25	0.57 (0.22–1.5)
aGvHD			
aGvHD ≥ grade 2	0.79	0.79	1.13 (0.45–2.8)
Source			
BM vs pBSC	0.035	0.090	0.17 (0.02–1.32)
BM or CB vs pBSC	0.95	0.399	1.13 (0.45–2.8)

HAdV: human adenovirus; HSCT: human stem cell transplantation; MFD: matched family donor; MMFD: mismatched family donor; MSD: matched sibling donor; UD: unrelated donor; aGvHD: acute graft versus (vs) host disease; BM: Bone marrow; pBSC: peripheral blood stem cells; CB: cord blood; OR: odds ratio.

[a] Effect coding: comparison versus all others.
[b] Reference coding: comparison versus a specific group.
[c] OR is not defined, lower limit of the 95% CI is given.

The median virus copy numbers in serial stool specimens observed within specific time intervals post-transplant in two different patient subsets, including individuals shedding HAdV already before HSCT and patients testing HAdV positive only after HSCT, are displayed in Fig. 1B. Intestinal HAdV shedding before transplantation revealed relatively low virus numbers in most instances, with a median of 9x10E3 HAdV DNA copy numbers/g stool as determined by RQ-PCR analysis (Fig. 1B). The median copy numbers in patients who tested HAdV-positive early post-transplant were 1–2 logs higher in most cases (Fig. 1 B), but the levels in individual patients were widely spread, ranging from 2x10E2 to 2x10E11 DNA copy numbers/g stool. In patients with HAdV shedding pre-transplant, rapidly rising virus levels exceeding the threshold of 10E6 DNA copies/g stool, which had previously been identified as critical for the risk of invasive infection (Lion et al., 2010; Jeulin et al., 2011), appeared already within 21 days after HCST (Fig. 1B). By contrast, the median virus loads in the stools of patients with first HAdV positivity only after HSCT did not reach this critical level of HAdV copies during the post-transplant course (Fig. 1B). Hence, the kinetics of intestinal HAdV levels in individuals with pre-

transplant virus shedding revealed a significantly earlier attainment of virus DNA copy numbers in stool above the indicated critical threshold ($p < 0.01$; Fig. 1B).

The characteristics of patients with HAdV shedding pre-transplant are displayed in Supplemental Table 1. The pre-transplant HAdV screening data revealed first positivity for the virus in stool before the start of conditioning in 35 of 42 patients, in line with the described association of HAdV persistence with intestinal virus shedding. However, HAdV screening of serial stool specimens in these patients including a median of three consecutive analyses (range 1–17) did not show positive results in all samples analyzed. The detectability of virus shedding in individual patients appeared to be intermittent, ranging from one to nine positive stool samples pre-transplant.

3.2. Time Point of First HAdV Detection in Stool and Risk for Invasive Infection in HSCT Recipients

The presence of invasive HAdV infection, defined by the onset of viremia, was observed during the first 100 days after transplantation in 33 of 304 (11%) children studied (Table 3), and occurred almost invariably in patients with prior detection of the virus in stool. The only exceptions included two patients in whom no stool samples were available shortly before and at the time of viremia onset, but stool specimens obtained within a few days after first detection of the virus in peripheral blood also tested HAdV positive, and revealed concordant species of the virus. Both univariate and multivariate analysis identified HAdV shedding pre-transplant as a risk factor for the development of viremia after HSCT (Table 2). The incidence of viremia in patients who revealed intestinal virus shedding before transplantation was significantly higher than in patients who did not (33% versus 7%; $p < 0.0001$; Table 3). Among the 42 patients with pre-transplant shedding of the virus, 32 (75%) remained HAdV-positive in consecutive stool specimens after HSCT, and this subset of patients displayed the highest incidence of viremia within 100 days post-transplant (14 of 32; 44%; Table 3). Two additional patients with intestinal HAdV shedding before transplantation developed viremia between days 100 and 140 after HSCT, raising the incidence of viremia to 50% when considering the extended observation time. The frequency of viremia was markedly lower in patients who tested positive for HAdV in stool only after transplantation (Table 3, subset D), although the difference between patient subsets E and D (Table 3) did not reach statistical significance. However, subset E included ten patients with HAdV positivity at a single time point pre-transplant, who had completely negative test results after transplantation. By contrast, subset F (Table 3) included only patients with more than one HAdV positive stool sample pre-transplant, and all of these patients also tested positive for the virus after transplantation. The difference between patient subsets D and F was statistically significant (44% versus 22%; $p = 0.019$; Table 3), revealing a particularly high risk for viremia in patients with persistent HAdV positivity in the stool pre- and post-transplant. Of the 87 patients with first HAdV detection in stool only after HSCT (Table 3, subset D), 19 (22%) developed viremia within 100 days post-transplant, and 17 of them had shown positivity in stool by day 56 post HSCT (Fig. 2). The onset of viremia occurred at a median of 21 days (range 0–92 days) after the first detection of HAdV in stool. In Fig. 2, the respective percentages of patients who developed viremia are indicated in relation to the first occurrence of HAdV positivity in stool within specific time frames.

In-vivo T cell depletion had been performed in 37 of 42 patients with HAdV shedding pre-transplant, and there was no apparent correlation with viremia. The HAdV levels observed in blood samples from patients with viremia ranged from 1x10E2 to 7x10E8 HAdV DNA copy numbers/ml, with a median of 1x10E4, in line with our earlier observations (Lion et al., 2010; Lion, 2014).
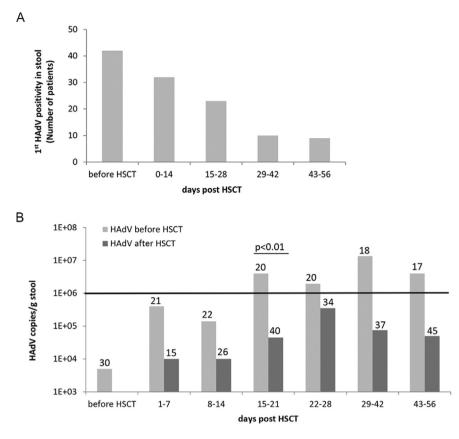
Fig. 1. First HAdV positivity and median virus levels in stool. A) Time intervals and the corresponding number of patients with first HAdV detection until day +56 post-transplant. Within this subset of 116 patients, 42 tested HAdV positive before, and 74 only after HSCT. B) Median values of HAdV DNA copies/g stool from patients shedding HAdV before HSCT (light grey bars) and from patients with first detection of HAdV positivity after transplantation (dark grey bars) are displayed. The virus copies were analyzed by RQ-PCR from stool specimens collected until day +56 post-transplant. The critical threshold for the risk of invasive infection at 1E + 06 HAdV DNA copies/g stool is indicated by the bold horizontal line, and the pertinent numbers of patient samples for each individual time point are indicated above the bars.

3.3. HAdV Species Distribution in Relation to First Detection of Positivity in Stool

Based on the notion that shedding of small HAdV amounts into the stool before transplantation reflects persistence of the virus in the GI-tract and detection of rising virus levels in stool specimens post-transplant is the result of viral reactivation, we have compared the occurrence of adenoviral species in both instances. All HAdV species detected before HSCT and during the first 100 days post-transplant were considered, including co-infections with more than one HAdV species in individual patients. Molecular analysis showed a concordant species distribution in stool samples of patients positive before or only after transplantation. A clear predominance of HAdV species C, followed by species A, and rather rare occurrence of other species were documented both pre- and post-transplant (Table 4).

4. Discussion

Invasive HAdV infections with disseminated disease still represent a life-threatening complication in the HSCT setting. Although primary HAdV infections have been documented in transplant recipients, the majority of cases apparently arise from reactivation of the virus, which can establish persistence upon primary infection (Kosulin et al., 2016a). Current data indicate that the GI-tract is the most important site of HAdV reactivation in the clinical context of pediatric HSCT. This notion is supported by the recently reported intestinal HAdV persistence in children (Kosulin et al., 2016a), and the observation that invasive infection in pediatric HSCT recipients is generally preceded by documented presence and expansion of the virus in stool (Feghoul et al., 2015; Lion et al., 2010). Additional evidence includes the similar prevalence of persistent HAdV in the GI-tract and the proportion of children with post-transplant virus reactivation, as well as the nearly identical distribution of adenoviral species in both instances (Kosulin et al., 2016a). The latter observation is in line with the highly concordant pre-and post-transplant occurrence of HAdV species in stool samples in the present study (Table 4). The observation of low median virus copy numbers in stool before transplantation (Fig. 1B) might reflect a persistent state of intestinal virus infection associated with low-level viral leakage into the stool, as
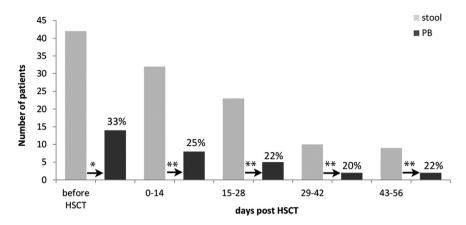
Table 3
Incidence of viremia.

HAdV in stool	Subset	No. of pts	Viremia[a]
HSCT recipients tested (total)		304	33 (11%)
Negative (total)	A	175	0 (0%)
Negative before HCST	B	262	19 (7%)
Positive (total)	C	129	33 (26%)
Positive only after HSCT	D	87	19 (22%)
Positive before HSCT	E	42	14 (33%)
Positive before and after HSCT	F	32	14 (44%)

Subset	p-Value	OR (95% CI)
A versus C	<0.0001	>7.3[b]
B versus E	<0.0001	6.4 (2.9–14.1)
B versus F	<0.001	9.9 (4.3–23.0)
D versus F	0.019	2.7 (1.7–6.6)
D versus E	0.16	1.8 (0.8–4.1)

HSCT: human stem cell transplantation; OR: odds ratio.
[a] The incidence of viremia within 100 days after transplantation.
[b] OR is not defined, lower limit of the 95% CI is given.

Fig. 2. Correlation between the time of first HAdV detection in stool and invasive infection post HSCT. The time intervals along the x-axis refer to the onset of first HAdV positivity in stool. The first HAdV detection in stool specimens (light grey columns) is displayed in relation to the corresponding frequency of viremia post-transplant (dark grey columns). The asterisks indicate the respective time spans between first detection of HAdV in stool and viremia, considering HAdV positivity in stool up to two weeks before HSCT: *median 35 days (range 8–92 days); **median 15 days (range 0–77 days). HSCT: hematopoietic stem cell transplantation.

indicated in our recent study (Kosulin et al., 2016a). Intestinal HAdV shedding before transplantation documented in the present study, which in most instances was evident prior to conditioning, further supports the notion that presence of the virus in the GI-tract appears to be in the state of persistence rather than latency. In patients with inborn or acquired severe immunodeficiency, intestinal shedding of the virus pre-transplant might reflect an early onset of reactivation. In individuals with most other underlying diseases providing an indication for HSCT, virus reactivation is apparently triggered by immunosuppression during the post-transplant period (Supplemental Table 1). Based on the present data, intestinal HAdV persistence associated with virus shedding into the stool prior to transplantation represents a risk factor for ensuing viremia which can be readily exploited in routine diagnostic monitoring in pertinent patient cohorts. It is conceivable that patients with persistent HAdV infection in the GI-tract represent the most important group at risk of reactivating the virus post-transplant, but the identification of viral persistence would require the analysis of intestinal biopsy material obtained by endoscopy, which may not be generally feasible in this clinical setting. However, molecular detection of HAdV shedding into the stool pre-transplant is an easily applicable non-invasive approach facilitating the identification of a subset of patients with persistence of the virus in the GI-tract carrying an elevated risk of systemic infection and disseminated disease. This observation corroborates earlier findings in smaller cohorts indicating that pediatric patients without detectable HAdV in stool apparently have a very low risk of developing invasive infection in the clinical setting of HCST (Kosulin et al., 2016a). Conversely, in line with earlier data, the risk of viremia is significantly increased in patients testing positive for the virus in stool (Fig. 2) (Feghoul et al., 2015; Lion et al., 2010; Jeulin et al., 2011). Children with documented intestinal HAdV shedding prior to HSCT displayed particularly rapid viral expansion in serial stool samples exceeding 10E6 copies/g, the critical threshold for the probability of invasive infection (Lion et al., 2010; Jeulin et al., 2011), already within 21 days post-transplant (Fig. 1B). These observations suggest that pre-transplant detection of HAdV in stool correlates with an elevated risk of early invasive infection, thus highlighting the importance of timely viral screening in stool specimens (Feghoul et

al., 2015; Lion et al., 2010; Jeulin et al., 2011). Moreover, the presence of critically high HAdV DNA copy numbers in stool very early after HSCT might also herald impending onset of invasive infection prior to incipient immune reconstitution, thus possibly predisposing for particularly severe clinical manifestations. Since early initiation of treatment was shown to be critical for successful management of HAdV infections in this clinical setting (Feuchtinger et al., 2006; Lion, 2014), the time point of first HAdV occurrence in stool specimens could be of relevance for appropriate therapeutic considerations. In this regard, pre-transplant HAdV detection in stool may provide the impetus for postponing elective transplantations and offering early pre-emptive treatment in high-risk settings. This strategy might be considered particularly in patients with more than anecdotal HAdV positivity in serial stool specimens pre-transplant, with rather high virus copy numbers already at this stage. Although this approach could result in the overtreatment of a considerable proportion of patients who might clear the virus even without therapeutic intervention, this option would appear reasonable provided that relatively non-toxic treatment options were available. It is conceivable that early initiation of therapy aiming at the prevention of invasive virus infection and disseminated disease might contribute to improving outcome. However, the risk of invasive infection must be judiciously balanced against the potential side effects of current treatment approaches. The availability of safer therapy options, such as the new compound Brincidofovir (Chittick et al., 2017; Hiwarkar et al., 2017), might facilitate the decision for early antiviral treatment once the efficacy of pre-emptive treatment is firmly established. Moreover, the timely employment and success of adoptive transfer of HAdV-specific T-cells using donor-derived (Geyeregger et al., 2014) or third-party cells (Arasaratnam and Leen, 2015; Eiz-Vesper et al., 2012) could be further improved by early recognition of high-risk patients in order to permit optimal planning and timing of T-cell generation or acquisition. The present study in a large cohort of pediatric patients provides evidence for the higher risk of viremia in individuals shedding HAdV into the stool prior to HCST, and greatly reinforces our earlier findings (Kosulin et al., 2016a). The current insights therefore provide a rational basis for further clinical studies on early pre-emptive treatment approaches to determine the potential impact on successful management of HAdV infections in highly immunocompromised patients.

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Table 4
HAdV species distribution in HSCT recipients pre- and post-transplant.

HAdV species	Before HSCT	Post HSCT
A	8 (15%)	21 (19%)
B	2 (4%)	8 (7%)
C	36 (68%)	66 (59%)
D	4 (8%)	11 (10%)
E	1 (2%)	4 (4%)
F	2 (4%)	2 (2%)

Potential Conflicts of Interest

T. Lion: Chimerix-consultancy and honoraria. The other authors declare no conflicts of interest in relation to the present study.

Author Contributions

KK: study design, data collection, analysis and interpretation, literature search, manuscript preparation, figure design and preparation. BB: data collection and analysis. SM, HP and AL: provision of samples, data collection. UP: statistical analysis, manuscript preparation. GF: data analysis and interpretation. TL: study design, data interpretation and manuscript preparation.

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