STUDY OF THE IMMUNE RECONSTITUTION IN PATIENTS RECEIVING A REDUCED INTENSITY STEM CELL TRANSPLANTATION FOR APLASTIC ANEMIA

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Ai miei genitori

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1. RIASSUNTO

L'Anemia Aplastica è una rara patologia ematologica, prototipo delle sindromi da insufficienza midollare, caratterizzata da un progressiva riduzione del patrimonio di cellule staminali midollari che esita nel classico quadro istologico di midollo "vuoto" o "grasso". Il quadro clinico è dominato dai sintomi tipici dell'insufficienza midollare, come astenia, petecchie e/o febbre, e può rapidamente deteriorarsi per l'insorgere di complicanze quali emorragie e/o infezioni, potenzialmente fatali. L'Anemia Aplastica colpisce generalmente 1-2/10^6 individui l'anno, con un'incidenza stimata nei paesi orientali di circa 2-3 volte superiore a quelli osservati in Occidente.

Sebbene l'etiologia rimanga sconosciuta nella maggior parte dei casi, in un quinto circa dei pazienti è riconoscibile una causa scatenante quale: esposizione a tossine (ad es. Benzene) e/o farmaci (ad es. FANS, sali d'oro, etc.) e/o agenti virali (ad es. Parvovirus B19, EBV, etc.). Raramente la diagnosi segue un episodio di epatite sieronegativa ed auto-limitantesi. A prescindere dal fattore scatenante, la patogenesi dell'Anemia Aplastica è immuno-mediata, ed è determinata dall'azione citotossica dei linfociti T che attaccano le cellule staminali ematopoietiche, determinandone la morte per apoptosi. Questo meccanismo autoimmune determina un difetto quantitativo e qualitativo delle cellule staminali, tipico della malattia. Recentemente tuttavia, la scoperta che le cellule staminali dei pazienti aplastici possono presentare telomeri corti, mutazioni dei geni del comlpesso telomerasico, e mutazioni in alcuni geni fondamentali nella regolazione epigentica del DNA, hanno suggerito che il danno funzionale del midollo osseo non derivi soltanto da meccanismi estrinseci immuno-mediati, ma anche intrinseci, derivanti da un primitivo deficit genico del comparto staminale.

La patogenesi immuno-mediata dell'Anemia Aplastica rende conto dell'alto tasso di risposte che i pazienti presentano ad un trattamento immunosoppressivo. Nel corso degli anni vari regimi di trattamento sono stati utilizzati, ma il più alto tasso di risposte è inevitabilmente associato all'impiego in combinazione di siero Anti-linfocitario (preferibilmente ATG di cavallo) e ciclosporina. Regimi alternativi, includenti Ciclofosfamide o Alemtuzumab, rimangono attualmente limitati all'impiego in trial clinici. Tuttavia la maggior parte dei pazienti non può definirsi completamente curata con l'immunosoppressione, per il consistente tasso di risposte incomplete e/o recidive riscontrato, e per il rischio di progressione clonale osservato anche nei pazienti responsivi. Per questi motivi, probabilmente riconducibili al primitivo difetto intrinseco staminale sopracitato, il trapianto di cellule staminali attualmente rappresenta l'unica terapia in grado di curare definitivamente questi pazienti.

Pertanto, in questo studio siamo andati ad indagare l'efficacia clinica e le dinamiche di ricostituzione immunologica di un regime di condizionamento ad intensità ridotta includente Fludarbina, Ciclofosfamide e Alemtuzumab (Condizionamento FCC) nel trattamento di questi pazienti. I risultati del nostro studio da un lato confermano gli ottimi risultati clinici del condizionamento FCC, per la ridotta incidenza di rigetto e GvHD, e la prolungata sopravvivenza globale e libera da eventi; dall'altro evidenziano una ricostituzione immunologica assolutamente peculiare, caratterizzata da una globale linfocitopenia post-trapianto nella quale 1) la rieducazione centrale dei linfociti T-CD4+, 2) l'espansione virus-mediata dei linfociti T-CD8+, 3) la presenza di cellule a fenotipo regolatorio (T-regs e B-regs) e 4) la costituzione di una condizione di chimerismo misto stabile creano una condizione di tolleranza post-trapianto refrattaria all'innesco di fenomeni immunologici potenzialmente nocivi (ad es. GvHD).

2. SUMMARY

Aplastic anemia is a rare hematological disease, prototype of bone marrow failure syndromes, characterized by a progressive reduction of bone marrow stem cells that leads to classic histological finding of “fatty” or “empty” marrow. Clinical picture is dominated by bone marrow failure symptoms, such as fatigue, petechiae and/or fever that can rapidly deteriorate to potentially fatal bleeding, or life-threatening blood sepsis. Aplastic Anemia commonly affects 1 to $2/10^6$ individuals per year, with an estimated incidence about 2-3 times higher in Eastern countries.

Although the etiology remains unknown in most cases, approximately one fifth of patients recognize a triggering event like: exposure to toxins (eg. Benzene) and / or drugs (eg. NSAIDs, gold salts, etc .) and / or viral agents (eg. Parvovirus B19, EBV, etc.). Rarely the disease follows an episode of self-limiting, sero-negative hepatitis. Regardless of this, pathogenesis of Aplastic Anemia is immune mediated, and is determined by cytotoxic action of T-lymphocytes against the hematopoietic stem cells, which eventually die by apoptosis. Due to this auto-immune mechanism, a quantitative and qualitative defect of stem cells is typically detectable. However, very recently, findings that stem cells of Aplastic Anemia patients may contain short telomeres, telomerase-genes complex mutations, or mutations in specific key genes of DNA epigenetic regulation system, have suggested that the functional damage of bone marrow does not derive only from an extrinsic immune-mediated mechanisms, but also form an intrinsic one, arising from a primitive genetic deficiency of stem cell compartment.

The pathogenesis of immune-mediated Aplastic Anemia realizes high response rate that patients show after immunosuppressive treatment. Over the years several treatment regimens have been experimented, but the highest rate of responses is inevitably associated with combinative use of anti-lymphocyte serum (preferentially horse ATG) and cyclosporine. Alternative regimens, including Cyclophosphamide or Alemtuzumab remain limited to clinical trials. However, the majority of patients cannot be defined completely cured with immunosuppression, for consistent rate of incomplete responses or relapses, and for the risk of clonal progression, present even in responding patients. For these reasons, probably related to the primitive intrinsic stem cell defect aforementioned, hematopoietic stem cell transplantation is currently the only chance of long-term cure in these patients.

In this study we investigated the clinical efficacy and immunological reconstitution dynamics of a reduced intensity conditioning regimen including Fludarabine, Cyclophosphamide, and Alemtuzumab (namely, FCC conditioning regimen) in the treatment of Aplastic Anemia patients. The results of our study clearly confirm excellent clinical results of the FCC-conditioning, for the reduced incidence of graft rejection and GVHD, and prolonged overall and event-free survival. Moreover, our result show clearly a peculiar immune reconstitution, where global post-transplant lymphocytopenia is associated with 1) central re-education of CD4+ T-lymphocytes, 2) virus-mediated expansion of CD8+ T-lymphocytes, 3) presence of cells with a regulatory phenotype (T-regs and B-regs), and 4) establishment of a stable mixed chimerism status, that all together institute post-transplant tolerance, refractory in triggering potentially harmful immunological phenomena like GvHD.

3. INTRODUCTION

3.1 Aplastic Anemia: from clinical features to pathogenesis of disease.

Disease definitions. Aplastic anemia (AA) is the paradigm of bone marrow failure syndromes. It's characterized by peripheral blood pancytopenia associated with an empty or fatty marrow as evidenced by bone aspirate and biopsy[1], which directly demonstrates the contraction of the hematopoietic compartment (Fig. 1). Even if most of cases remain idiopathic, a large amount of data support the idea that the empty bone marrow is determined by an auto-immune disruption of hematopoietic stem cell (HSC), which leads to deficient hematopoiesis and disease related symptoms, defining AA as an autoimmune disease. Acquired forms of AA are far more frequent than constitutional ones, and typically affect young adults or elderly people, who present with peripheral pancytopenia in absence of other hematological diseases. The typical patient, young and previously well, presents with symptoms of mild bleeding and easy fatigue, but less frequently AA starts as fulminant illness, marked by continuous infections and recurrent hemorrhages, with a life-threatening condition[2]. Specific criteria define severity of disease (Moderate, severe or very severe)[3] according to number of peripheral blood cytopenias and bone marrow cellularity (Table 1).

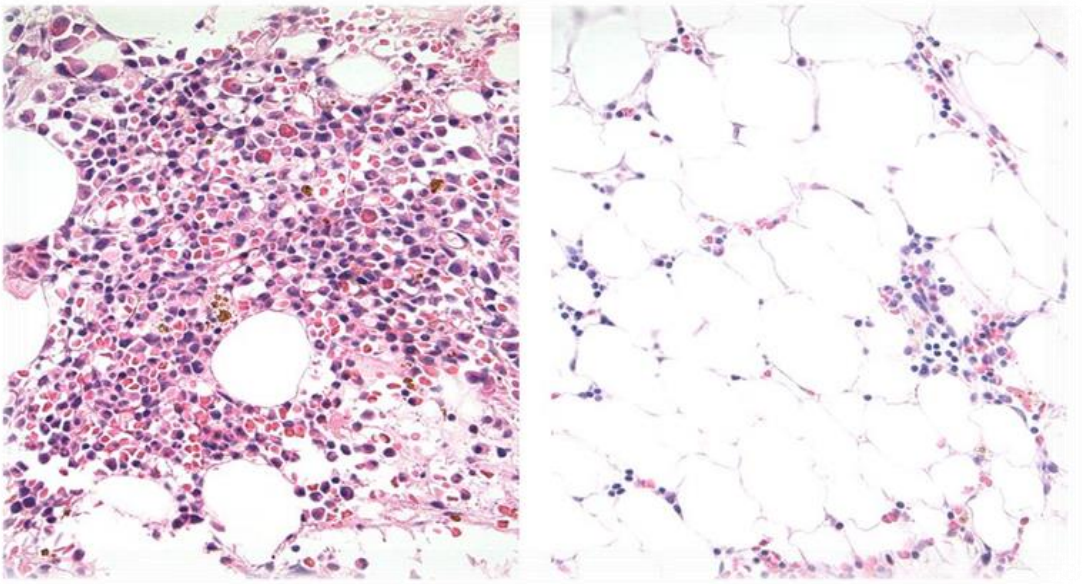

Fig.1. Normal bone marrow (Left side) compared with AA marrow (Right side)

Disease epidemiology. In almost all modern studies, sex ratio has been close to 1:1, which remains quite unusual for an immune mediated disease. Age of incidence shows a typical bimodal peak of distribution, with most of patients receiving diagnosis between 15 and 25 years, or over 60 years of age. Population-based studies have clearly established that disease incidence is relatively low, with an estimated rate of 2-5 person affected per million per year[4].

Classification	Criteria
Severe	BM cellularity $< 25\%$ (or $< 50\%$ if $< 30\%$ of BM is hematopoietic cells) AND ≥ 2 of the following: • Peripheral blood neutrophil count $< 0.5 \times 10^9$/L • Peripheral blood platelet count $< 20 \times 10^9$/L • Peripheral blood reticulocyte count $< 20 \times 10^9$/L
Very severe	As above, but peripheral blood neutrophil count must be $< 0.2 \times 10^9$/L
Nonsevere	Hypocellular BM with peripheral blood values not meeting criteria for severe aplastic anemia

Table.1. AA classification according to severity of disease[3].

However a marked geographic variation in disease incidence has been observed. In Europe and Western countries, a recent survey by Montanè et al (2008)[5] has reported a incidence of 2.34/million that is similar to the rate of 2.0/million showed by the International Agranulocytosis and Aplastic Anemia Study in Europe and Israel (IAAAS, 1985)[6], and to smaller national studies lead in France[7], United Kingdom[8], Scandinavia[9] and Brazil[10]. In Asia and Far East countries, incidence of AA appears to be much higher: a large cohort study from Thailand found a rate of 3,9/million in the urban area of Bangkok[11] and of 5/million in the rural area of Khonkaen[11]. Similarly a prospective survey of Chinese Epidemiologic Study Group of Leukemia and Aplastic Anemia[12] reported an even higher rate of 7,4/million, showing how AA can rival with Acute Leukemia as admitting diagnosis in Haematological divisions of Chinese hospitals. Therefore, AA incidence appears to be 2- to 3-fold higher in Eastern than Western countries, with a frequency which hasn't shown significant modifications across years.

Etiology – Toxins and drugs. Even if most of cases remain of unknown origin, since the first case of AA was described an enormous amount of data on precipitating or causative factors has been accumulated in medical literature. Cytotoxic drugs and radiation are the best examples of factors that can directly injury HSC. In fact, although stem cells are more resistant to cytotoxic drugs, for most agents a dose-response relationship with the degree of stem cell damage can be established. Moreover, different studies have identified a list of chemicals and drugs whose exposure is associated with AA onset. Benzene[13], for example, is the most widely studied and implicated amongst chemical toxins. The relationship with AA was initially brought to light by case series of workers exposed through their specific occupation: studies on American workers earlier in this century suggested that risk of AA was about 3% in men exposed to concentration higher than 300 ppm[14], and in the more recent IAAAS[6] study benzene was accounted for about 1-3% of AA recorded cases. Specific drug associations have been established in different population-based study and have changed across time, mainly due to changes in drugs diffusion and utilization. Association with Chloramphenicol[15], for example, that gained notoriety in the 1950s, and that for decades was considered the commonest cause of the disease, has progressively declined to the point that it has not been reported as significant risk factor in any recent systematic epidemiologic study of AA in Western countries[5]. Nowadays a long list of drugs associated with disease onset is available,

but mostly commonly seen associations are reported with gold salt (Relative Risk, RR of 29), anti-thyroid drugs (RR of 11), and Non-steroidal Anti-inflammatory Agents (RR of 8.2 for Indomethacin)[15].

Etiology – Viral infections. Among the potentially etiologic associations between environmental exposure and marrow failure other than drug or chemical toxins, viral infections have been linked to AA pathogenesis too. Parvovirus B19[16], the causative agent for Fifth disease in the immunocompetent host, is associated with transient aplastic crises in chronic hemolytic anemias such as sickle cell disease, and cases of severe AA have also been reported in normal individuals during an acute episode of infection. More generally, cases of AA associated with HAV[17], HBV[18], HGV[19], Epstein-Barr virus (EBV)[20], or echovirus[21] infections have been reported. Very recently retrospective observations about eight AA cases occurring during HIV[22] infection have been reported, suggesting that AA could be a late rare complication of HIV infection too. Post-hepatitis AA[23] is a stereotypical syndrome, where pancytopenia often presents two to three months after an acute attack of sero-negative, severe but self-limited, liver inflammation. This distinct variant has been commonly seen in 5-10%[24] of 'classical' AA cases, typically occurring in adolescent boys and young men. The viral agent responsible for this syndrome has not been identified, but it is possible that at the time of overt cytopenia the viral infection is already cleared, with AA onset being mediated by lymphocytes recognizing a cross-reactive antigen in the marrow. Effective response to immunosuppressive therapy strongly suggest this immune mediated mechanism.

Immune pathophysiology. Even if a putative inciting agent directly attacks the stem cell pool, causing a permanent depletion of HSC, generally the clinical presentation of cytopenia may be delayed for weeks or months, appearing just when a critically low stem cell number is reached. Similarly, an indirect damage of HSC can be sustained by an immune effector mechanisms, which may be triggered by viruses or by drug metabolites, and which may take time before becoming clinically evident. An analogous mechanisms may be postulated in the majority of cases of idiopathic AA, where an unknown initiating agent, probably through presentation or cross-reactive triggering of neo-antigens, results in a breach of immune tolerance and in generation of an immune-mediated attack towards hematopoietic progenitors, leading to HSC consumption and functional impairment with subsequent pancytopenia (Fig. 2a)[25].

The responsiveness to immunosuppressive therapies (IST) remains the best evidence of an underlying immune pathophysiology of AA. In early laboratory experiments, removal of lymphocytes from aplastic bone marrows improved colony numbers in tissue culture, and their addition to normal marrow inhibited hematopoiesis in vivo[26]. Even if early stages of autoimmunity in AA have been less characterized, a large amount of studies have provided clear data on proximal events of immune-mediated marrow failure. The effector cells were identified by immunophenotyping as activated cytotoxic T-lymphocytes expressing Th1 cytokines, especially Interferon-γ (IFN-γ). CD8+ T-cells containing intracellular IFN-γ can now be measured directly in peripheral blood of AA patients[27], and oligoclonal expansion of CD8+CD28- T-cells, defined by flow cytometric analysis for T-cell receptor (TCR) Vβ subfamilies[28], by spectratyping to detect skewing of Complementary Determining Region-3 (CDR3) length, and by sequencing of the CDR3 region to establish a molecular clonotype, has been detected in AA patients. In general, at presentation of disease patients demonstrate oligoclonal expansion of few Vβ subfamilies, which diminish or disappear with successful therapy. Original clones re-emerge with

relapse, sometimes accompanied by new clones. Very occasionally a large clone persists in remission, suggesting establishment of immune tolerance (Fig. 2b)[25].

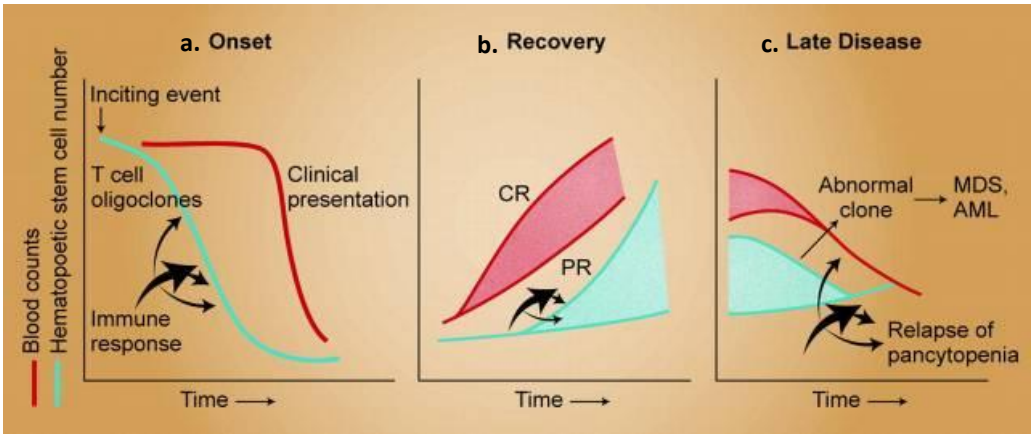

Fig.2. Interactions between blood counts, immune system and AA course. Modified from Young et al[25].

T-lymphocytes stem cell damage is mediated by direct cell-to-cell killing as well as by cytokine induced inhibition; the latter is documented by excess production of type I cytokines, especially IFN-γ and Tumor Necrosis Factor-α (TNF-α). T-Bet is a transcription factor that binds IFN-γ promoter and induce interferon genes translation in T-lymphocytes. Patients with AA show constitutive high expression of T-Bet gene[29]. Additionally, IFN-γ and TNF-α activate the Fas-ligand[30] and Tumor Necrosis Factor-derived Inhibitory Ligand (TRAIL)[31], which play an important role in HSC inhibition by direct cell-to-cell adhesion. Such mechanisms may not be restricted to the primary target only, but may also attack innocent bystander cells, like stromal cells. Ultimately, these factors result in apoptosis[32] of all existing bone marrow cells, through induction of different mechanism like Interferon Regulatory Factor 1 (IRF1), inducible NOS (iNOS) and production of nitric oxide (NO), and Fas- and TRAIL-dependent pathway of cell death. Finally, IFN-γ, TNF-α and Fas-L modulate the expression of their receptors through feedback mechanisms[33, 34], and in this way they may enhance each other action (Fig. 3)[25].

Few putative auto-antigens have been identified as potential target of autoimmunity in AA, mainly through patients sera antibodies screening against human peptide library. Kinectin[35], a widely expressed protein of cytoskeleton, bound to antibodies from about 40% of AA patients. In a smaller minority of patients, Diazepam-related Binding Protein-1 (DRBP-1)[36], an enzyme essential for oxidation of fatty acid and distributed in different tissues, has been identified with same methodology. Which relevance in an essentially T-cell mediated pathology have these autoantibodies remain unclear. However, for Kinectin reactive cytotoxic T-cells can be generated in vitro and inhibit human HSC. Recently, a novel, autoreactive, CD1d-restricted, GPI-specific T-cell population[37], enriched in a specific invariant TCRα chain (Vα21 sequence), has been documented in Paroxysmal Nocturnal Hemoglobinuria (PNH) patients. Since GPI-negative (not expressing surface protein with a Glycosyl-Phosphatidyl-Inositol anchor) hematopoiesis represents an escape mechanism to bone marrow failure in the pathogenesis of PNH, this finding suggests a potential role for a putative GPI-linked auto-antigen in the pathogenesis of acquired BMF in PNH[37].

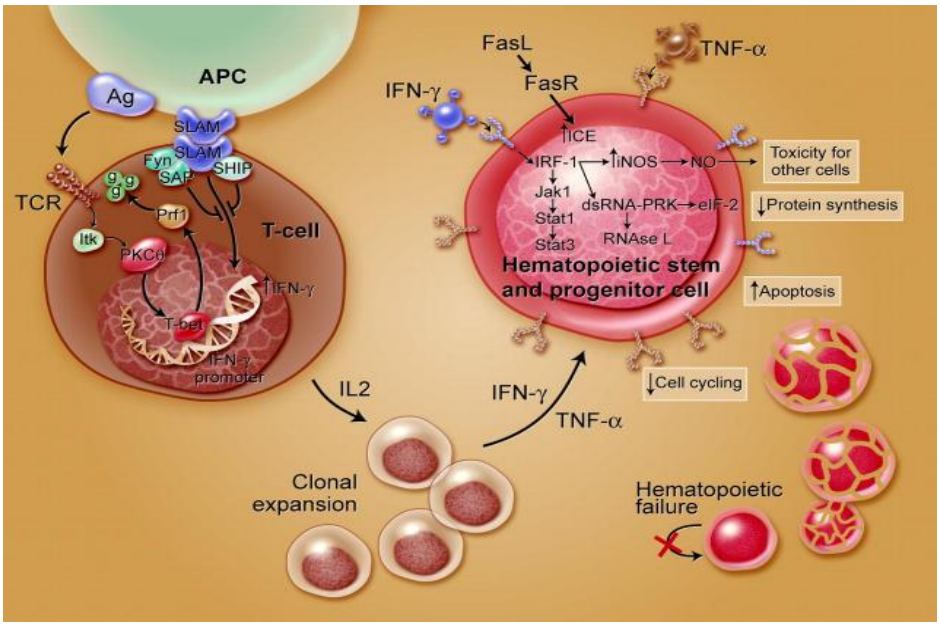

Fig.3. T-cell activation and HSC killing in AA.
Modified from Young et al[25].

Dysfunction of HSC in AA. Regardless the nature of injury, AA patients are characterized by a severe dysfunction of the HSC compartment; this defect has been deeply characterized in a both quantitative and qualitative fashion. Patients with acquired AA show a very low number of hematopoietic progenitors, as measured by flow cytometric CD34+ cell assessment or by in vitro colony assays[38, 39, 40]. By flow cytometry, CD34+ cells are reduced in all AA patients, and the contraction affects both committed, and immature CD34+/c-kit or CD34+CD38- progenitors[41]. The minimal number of colonies derived from committed progenitors in semisolid media reflect primitive HSC defect in these patients. Long-term culture-initiating cells (LTC-IC) as well as cobblestone forming assay have been developed as surrogate of HSC activity assays[42-44]. The LTC-IC assay assesses cells capable of colony formation after 5 weeks in long-term bone marrow culture, and share the frequency, phenotype, and kinetic properties of true stem cells. Several studies indicate a profound deficiency in LTC-IC as well as cobblestone unit initiating cells in all patients with AA. At the time of clinical presentation, the number of LTC-IC is usually at least one log below the normal level[45]; combined with a reduction in total marrow cellularity to <10%, the stem cell number in AA is estimated to be reduced at least of two logs compared to healthy individuals[45]. Neither the LTC-IC number, nor that of colony forming cells, correlate with the blood counts, suggesting that in addition to the quantitative defect, a functional impairment may be present; this may also be extrapolated by the observation that the clonogenic capacity of an individual progenitor is lower than in normal. The reduced clonogenicity was demonstrated both on CD34+ cells[46] (as number of colonies obtained from a purified CD34+ population) and on the putative stem cell LTC-IC (as number of secondary colonies assayed from LTC-IC in limiting dilution experiments)[47].

HSC compartment is affected by the pathophysiologic process operating in AA, whereas mesenchymal or even more immature pluripotent stem cells are likely functionally normal. Indeed, several studies[48-50] have documented that marrow cells from AA patients are able to generate in vitro perfectly functional stromal layers, as

confirmed on cross-over experiments, which strongly support that stromal progenitors or early pluripotent stem cells are not affected in AA. This is also confirmed by the clinical observation that allogeneic stem cell transplantation is a highly successful therapy for AA, even if most stromal elements remain of host origin.

Clonal events in AA. In last ten years several finding have enriched our knowledge of primitive HSC damage in AA, suggesting that the quantitative and qualitative defect exhibited by CD34+ cells may be not only related to the extrinsic attack of the immune system in bone marrow microenvironment, but may reflect also a primitive and potentially intrinsic genetic defect of HSC. First evidences of this conditions emerged from clinical finding that 10% to 15% AA patients can progress[51, 52] from a not malignant condition to clonal disease like PNH and Myelodisplastic Syndrome (MDS) or less frequently Acute Leukemia (Fig. 2c). Fifty percent or more of patients with AA have expanded PNH cells at diagnosis; most clones are small and do not lead to clinical symptoms of hemolysis or thrombosis, but in some cases classic PNH can be dominated by bone marrow failure symptoms, depicting the AA/PNH syndrome[53]. The large absence of GPI-linked surface protein in PNH cells, caused by mutation occurring in PIG-A gene sequence, which codify for an enzyme necessary for ligation of cell-surface protein to a GPI anchor, has generated the hypothesis that expansion of PNH clone could represent an escape mechanism to the immune attack responsible for of AA pathogenesis[37]. Indirect evidences for this derive from experimental observation that in AA patients PNH clone derived CD34+ cells preserve a proliferative capacity and does not express Fas-L, when compared with “normal” CD34+ cells[54].

Moreover, stereotypical patterns of aneuploidy have been observed in AA patients over time: monosomy 7 or trisomy 8 are the most characteristic, while less frequently translocation involving chromosome 3 and 13 are observed too[55]. Even if these anomalies can be rarely present at diagnosis, when differential diagnosis between AA and MDS with hypoplastic features may become challenging, their occurrence is generally observed as late event in AA history, from 10 to 15 years after initial diagnosis, sometimes even after a successful treatment with immunosuppression. In these case appearance of a cytogenetic abnormality is almost inevitable linked to clinical consequences, like profound progressive pancytopenia, leading to MDS and/or eventually to Acute leukemia (Fig. 2c)[25].

Telomere length in AA. Very interestingly, a significant telomere shortening has been reported in patients with AA, and telomere length in the lowest quartile has been correlated with a major risk for clonal evolution in general, and for monosomy 7 in particular, with an estimated rates 5 to 6 times higher than in patients where telomere content is higher[56]. Telomeres are hexanucleotide (TTAGGG) repeats at chromosome ends, organized in secondary structure termed the T-loop, and associated with a protein complex collectively termed Shelterin. Telomerase is the ribonucleoprotein enzyme complex that synthesizes telomeres, that includes a reverse transcriptase, (encoded by gene TERT), an RNA template (encoded by gene TERC), and associated proteins that affect assembly, trafficking of Telomerase to telomeres, and Telomerase stability, such as Dyskerin (Fig 4). The cell is sensitive to critically short telomeres, which trigger DNA damage responses and lead to cell senescence or apoptosis, defining telomere length as a “mitotic cell clock” critical for survival. Telomeres erosions occur normally with aging, as well as under environmental circumstances where a regenerative stress and/or oxidative damage is present[57]. Accelerated telomere shortening and attrition seen in AA may be a result of replicative stress imposed to the few remaining HSC after selective pressure

derived from immune system attack. Given this, telomere driven genomic instability would lead to chromosome loss and haplo-insufficiency of genes as on chromosome 7, determining disease progression to MDS and eventually leukemia[58].

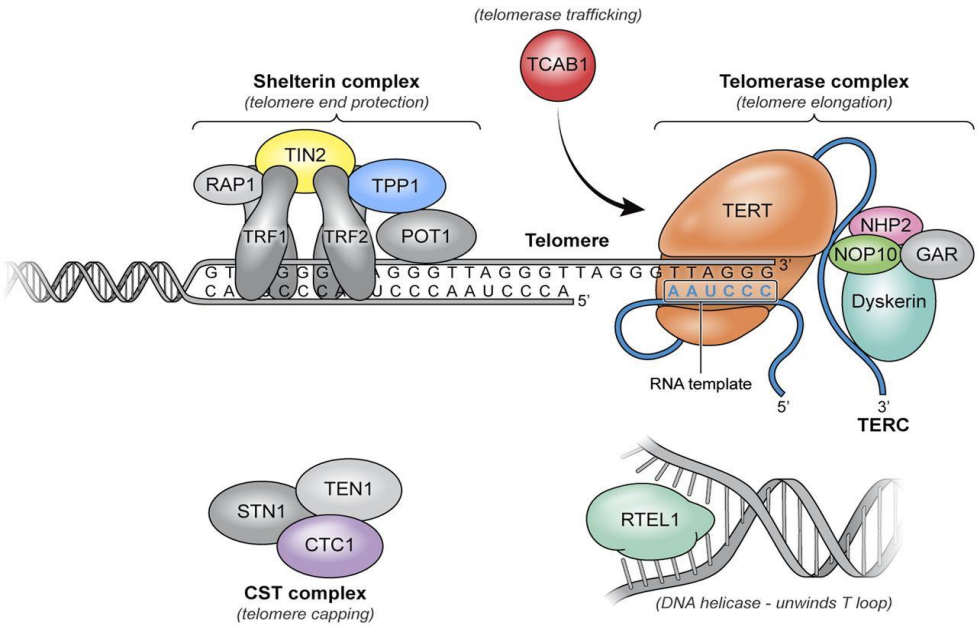

Fig.4. The Telomerase / Shelterin complex.

However, marrow cells from patients collected years earlier before evidence of clonal disease may present both telomere loss and chromosomal aberrations many months or years prior of clinical evolution to MDS/AL. Intriguingly TERC, TERT and DCK1[59,60] mutations can be found in these individuals, making the historically clear distinction between constitutional and acquired aplastic anemia blurred due to the presence of mutations otherwise typical for congenital bone marrow failure syndromes (i.e. Dyskeratosis Congenita) in adult cases with no apparent family history and lacking classic physical anomalies. In these cases, telomere shortening may reflect defective ability of HSC to respond to stress due to intrinsic genomic deficiency in Shelterin/telomere complex, rather than a late clinical picture of a congenital disease.

Gene mutations in AA. Finally two[61,62] different groups have recently identified tanks to Next-Generation-Sequencing (NGS) analysis evidence for clonal hematopoiesis in AA. The majority of the mutations detected occur in genes involved in epigenetic regulation of DNA transcription, like ASXL1, DNMT3A and TET2, or in different genes like BCOR/BCOR-L1 and PIG-A. Various sets of mutations show distinct behavior and clinical effect; BCOR, BCOR-L1 and PIG-A mutant clones tend to disappear or remain small across time, are associated with a better response to IST, and predicted favorable outcomes. In contrast, clones carrying mutations in DNMT3A, ASXL1 and few other genes are more likely to increase in size over time, and are associated with a poorer response to IST, inferior overall survival, and with progression to MDS, Acute leukemia, or both. Very interestingly, these findings couple with recently published data[63,64] showing that clonal hematopoiesis may occur in healthy individuals with older age, probably as result of Darwinian process which

select clones with survival advantage in the contest of ageing myelopoiesis. The presence of similar mutated clones in AA could indicate clonal hematopoiesis as a selective adaptation in the context of immune subversion/pressure secondary to disease onset.

More intriguingly, data on clonal evolution, telomere length and gene mutation has finally started to depict a diversified and complex pathogenetic process, for which AA has to be seen not simply as simple autoimmune disease, but as complex stem cell disease where overlapping areas and gray-zones with other clonal malignant hematological disease exist (Fig 5).

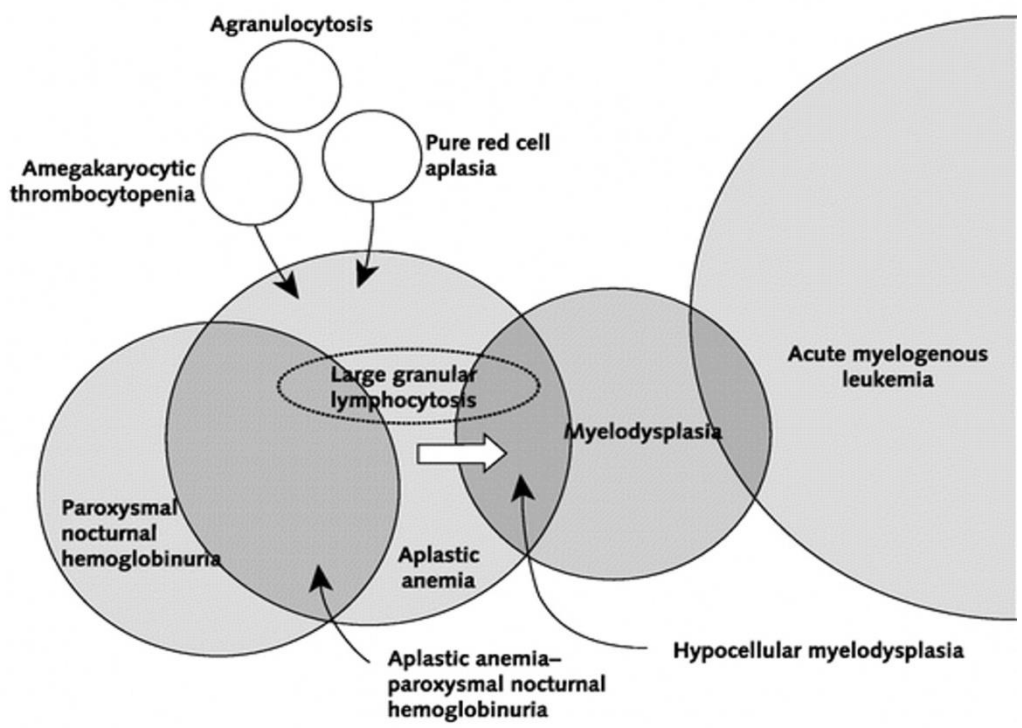

Fig.5. Venn diagram showing overlappin between AA and other clonal disease.

3.2 Current concepts on treatment of Aplastic Anemia: focus on immunosuppressive therapies

Standard IST regimen in AA. The efficacy of immunosuppressive treatment (IST) in AA was firstly discovered in the early 60s, when observations from Mathè et al. highlighted how autologous recovery was possible in patients receiving Anti-Lymphocyte Globulin (ALG) as part of conditioning regimen for allogeneic bone marrow transplant. After this report, two important randomized trial showed clearly the superiority of ALG based therapy on oral androgens and on supportive therapies in not transplanted patients, setting antiserum therapy as the standard IST for AA[65].

The subsequent aim for clinicians was to obtain an increased response rate and a sustained response, preventing subsequent relapses, combining antiserum therapy with ALG or Anti-Thymoglobulin (ATG) with other immunosuppressant agents, like corticosteroids (i.e. methylprednisolone), androgens and cyclosporine-A (CyA). Only CyA, a calcineurin/NF-Kb inhibitor which impairs interleukin IL-2 dependent T-cell activation and differentiation, has proven to be effective in these patients, as initially demonstrated by the German Aplastic Anaemia Group in a randomized trial[66]. In fact, the addition of CyA to ATG and steroids (utilized mainly for serum-sickness prophylaxis) increased the 6-month overall response rate from 46% to 70% in AA patients, possibly impacting long-term survival (even if no statistically significant difference was shown in this initial experience). After this study, and from 90s on, ATG + CyA has become the most utilized IST for AA patients, with a reported response rate ranging between 50 to 65%, and with an expected overall survival rate at 1-year of 60%[25]. Even if in the majority of available studies, the overall response rate [complete response (CR) + partial response (PR)] does not exceed 70%, and has remained stable in the last twenty years, most recent studies have shown improved overall survival (above 80% at 1-year), independently from the initial response to IST[67]. Probably this effect has to be correlated with the improved supportive care, and better timing for salvage treatment, especially stem cell transplantation, that inevitably have an impact on long term overall survival.

Impact of ATG type on IST results. An important point that needs to be clarified in antiserum therapy is that ATG/ALG is a heterologous anti-serum obtained by injecting human lymphocytes into animals. Therefore various ATG preparations exist, which can differ for stimulating antigens (peripheral lymphocytes, thymocytes, or even T-cell lines), and/or for host animal (either horse or rabbit). Most importantly, this differences hesitate in biological and clinical differences. The most dramatic biologic differences detected is the anticipated and more profound lymphocyte depletion observed in patients treated with rabbit ATG (r-ATG) compared with ones exposed to horse ATG (h-ATG), particularly for CD4+ T-cells, and including CD4+ T-regulatory elements[68]. However, if this differences represent simply an association, or instead an explanation for h-ATG superiority in the clinical setting, remains still a matter of debate, especially if we consider that r-ATG was shown to be effective in rescuing a proportion of patients who previously failed h-ATG therapy.

Most available data from large randomized clinical trials refer to polyclonal h-ATGs, which have to be considered the gold standard for AA treatment. US and Japanese investigators utilized hATG (40 mg/kg per day, for 4 days; ATGAM; produced by Pharmacia US, or PFIZER Pharma), which is different from the h-ATG previously used in Europe (15 mg/kg per day, for 5 days; Lymphoglobuline; produced by Genzyme, or Fresenius Biotech). In the National Institutes of Health (NIH) experience[69], the 3-year probability of survival was 96 % for h-ATG, versus 66 % for r-ATG, with a higher incidence of early deaths for infections in the r-ATG group. Similarly, the European Blood and Marrow Transplantation (EBMT) Group study[70], which compared patients receiving r-ATG to matched historical controls treated with h-ATG (Lymphoglobuline), confirmed these differences, with h-ATG patients having a 2 year-OS of 86% instead of 68% r-ATG patients survival rate. Even in the EBMT study, an higher incidence of fatal infection was observed in the r-ATG group, although at a later time-point (after day +100), when compared to the NIH study. Interestingly, a retrospective study[71] on a Japanese population patients receiving r-ATG showed similar response rates to the ones who received h-ATG treatment.

However, these results were not significant and 2-year, while at 10-year observed overall survival was again superior in the h-ATG group.

Despite these evidences, h-ATG was withdrawn from the market after 2007 in Europe, leaving r-ATG, that formerly was a second-line choice in patients failing to respond to h-ATG, as the only available first line ATG for AA. Two r-ATGs are currently commercialized in Europe, but to the date clinical results with these agents are less robust for the lack of large randomized trials. In most cases, r-ATG was used as second-line IST (after initial h-ATG) to prevent side effects due to possible sensitization to horse proteins. In these setting, r-ATG resulted in an overall response rate up to 68% (in relapsed patients), suggesting an alternative use even in first line, whenever h-ATG preparation is not available.

Intensification of standard IST in AA. Several attempts have been made to explore intensification of standard IST to improve response rates, increase stability of response and therefore reduce relapse rate. Essentially these trials explored two different options: 1) addition of a third immunosuppressant drug to the standard ATG+CyA regimen, or 2) usage of an alternative regimen including drugs different from Antiserum therapy.

Intensification of immunosuppression has been explored adding high-dose corticosteroids, mycophenolate-mofetil (MMF), or rapamycin to standard treatment with h-ATG. Despite methylprednisolone at very high dosage (20 mg/kg/day) has shown some activities in AA patients, this condition did not show any improvement in response or survival rates. MMF, a purine synthesis inhibitor that is currently used both in bone marrow and solid organ transplantation to prevent GvHD or graft rejection, was tested in a prospective study conducted at NIH. Addition of MMF[72] to ATG+CyA did not confer any benefit on either response rate (62% at 6 months) or relapse rate (37%, despite maintenance therapy with MMF) in comparison to historical data. Again, investigators at NIH assessed the potential benefit in AA of rapamycin/sirolimus (RAPA)[73], a mammalian target of rapamycin (mTOR) inhibitor, which shares with CyA ability to block initial IL-2-dependent T-cell activation, and in addition to CyA is able to inhibit mTOR downstream signaling, affecting several intracellular pathways essential for cell-cycle progression and T-lymphocytes survival. In a randomized prospective trial, the addition of RAPA to the standard ATG+CyA regimen did not result in any improvement of haematological response in comparison to the control arm. One-year overall response rates observed were respectively 51% and 62%, with comparable relapse and survival rates in both arms.

Alternative IST have been experimented with regimens including Cyclophosphamide and Alemtuzumab. A single phase 2 study conducted at the Johns Hopkins University testing high-dose Cyclophosphamide (50 mg/kg/die x 4 days) for treatment-naïve AA patients was recently updated (2010)[74]; this study reported an actuarial overall survival and overall response rate respectively of 88% and 71%, with an actuarial event-free survival rate of 58% at 10-years. Unfortunately this treatment was associated with a long-lasting neutropenia, and severe fungal infections occurring at a rate of 18.2%. Similarly, in a randomized study conducted at NIH, directly comparing high-dose Cyclophosphamide to standard IST with h-ATG+CsA, an excess of toxicity and death from invasive fungal infections were observed in the Cyclophosphamide arm, leading to an early termination of the study. A Chinese trial of “moderate” doses of Cyclophosphamide (30 mg/kg/die x 4 days) appeared attractive for the abbreviated period of neutropenia, little morbidity and mortality, and a response rate comparable to ATG + CyA regimen. Those results, combined with the recent drastic improvement of antifungal therapies justified further

investigation of moderate doses of cyclophosphamide. Unfortunately, when Scheinberg et al[75] at NIH reported results of “moderate-dose” Cyclophosphamide (120 mg/kg) plus low-dose CyA in AA untreated patients, again the safety monitoring board of study recommended termination due to unacceptable toxicity, for excessive length of severe neutropenia (<0.2 × 109/L), and excessive rate of fungal infections, despite usage of aggressive antifungal prophylaxis. These data states clearly that Cyclophosphamide is not a competitive agent for the treatment of AA outside of clinical trials.

Alemtuzumab, a monoclonal antibody that is able to induce lymphocyte depletion through specific killing of CD52-positive cells via both antibody-dependent cellular cytotoxicity and complement-mediated lysis, is another alternative immunosuppressant agent that have been explored in treatment of AA patients. A report from Risitano et al in collaboration with the EBMT has shown that an Alemtuzumab-based IST[76] is feasible, safe and effective treatment option in AA. Preliminary data suggest a response rates not below standard IST regimen (58%), with easy re-treatment in case of relapse. Moreover, and differently from Cyclophosphamide, an acceptable rates of infections and toxicities has been observed.

3.3 Allogeneic Stem cell Transplantation for Aplastic Anemia

Rationale for Stem Cell Transplantation in AA. There are several reasons for which allogeneic hematopoietic stem cell transplantation (HSCT) at the current state remains the only definitive curative option in AA. First of all, even if most AA patients achieve a clinical benefit from immunosuppression, the quality of response is generally heterogeneous, with blood counts that could stay below normal values in responding patients too. With current IST regimens two third of patients shows a response after immunosuppression, that generally is equally distributed between CR and PR, but most of them (ranged between 25% and 50%) keep requiring long-term lower doses of CyA to maintain stable blood cells counts, and cannot be considered totally cured. Even when patients show a complete response, relapses are frequently seen, with a range in time that varies from months to years from IST discontinuation[25].

Secondly, about one third of patient doesn’t show any response to current standard h-ATG+CyA treatment and results to be refractory to IST; in these patients re-challenge with a second round of IST generally hesitate in dismal outcome for suboptimal responses and higher rates of infections. Molecular evidence on telomere length and gene mutations, as aforementioned above, suggest that in this category of AA a primitive intrinsic HSC damage develops early and persists independently from immune-dysfunction. In according with these observations, risk progression to clonal disease is not abolished after IST treatment, even when successful, and remain particularly higher in patients failing IST[51,52].

Given this, allogeneic HSCT remain the only long-term curative option able to guarantee a complete recover of bone marrow function in a disease where the stem cell compartment remain partially or totally dysfunctional even after IST.

Allogeneic HSCT has been proposed as a potential treatment for AA since the early 60s, when initial reports described successful transplantation from geno-identical twins (syngeneic HSCT)[65]. However, even in this setting became

immediately clear that intensity of immunosuppressive conditioning was able to increases chance of engraftment; therefore in subsequent years several attempts were made to intensify immune suppression in HSCT and finally ameliorate HSCT outcome. As result of this constant improvement, and based also on the outcome of IST, HSCT from an HLA-identical sibling donor (SIB) is now considered the treatment of choice for young AA patients[65]. Moreover, higher-resolution HLA-typing in recent years allowed a better donor selection and resulted in a significant improvement of survival even after HLA-matched HSCT from unrelated donor (MUD), making feasible access to HSCT even for patient where a familial donor was not available. Novel strategies of immunosuppression also allowed HSCT across the HLA-barrier, and HLA haplo-identical HSCT (Haplo) has proved to be successful in small series of AA patients. Finally, HSCT from cord blood (CB) units remains an alternative option for AA patients lacking a HLA-identical/well-matched donor.

Irrespective of the feasibility of HSCT from different donor types, HLA-matching remains the most relevant factor affecting the outcome in AA. In addition to this, success of allogeneic HSCT in AA is also largely affected by some transplant-related factors, which include patient age, stem cell source, conditioning regimen, T-cell depletion, graft rejection and graft-versus-host disease (GvHD).

HSCT from HLA-identical SIB donor. Allogeneic HSCT from HLA-identical SIB donor result in long-term high survival rate in young AA patients. The standard procedure has been settled based on prospective studies carried mostly from the Seattle's group, who explored in the context of HSCT for AA a conditioning regimen with high-dose cyclophosphamide (CY; total 200 mg/kg fractioned in 4 doses). The benefit of addition of ATG to CY (namely, Cy200-ATG conditioning) has been investigated in a prospective randomized trial, which enrolled 134 AA patients undergoing T-cell replete HSCT from sibling donors. Even if the CY200 + ATG arm showed a slight improvement in overall survival (80 vs 74% at 5 years), this was not statistically significant, likely due to not adequate statistical powering of the study[77]. Thus, the evidence to support the combination of CY200-ATG as conditioning regimen comes mainly from previous non-randomized trial[78], where this regimen resulted in improved survival with reduced chronic GvHD rates, as compared with historical controls who have been conditioned by CY alone. However a clear point that need to be specified is that current survival reported with Cy200-ATG range from 65% to 95%[78,79], according to patient age, and that this conditioning regimen is associated with a very low incidence of second tumors and preservation of fertility.

Alternative T-cell depletion with lymphodepleting agent other than ATG like Alemtuzumab have been explored, and actually the role of in vivo conditioning with ATG or Alemtuzumab for HLA-identical SIB HSCT remain a controversial point of debate. A randomized trial failed to show a survival advantage for patients receiving ATG in the conditioning regimen when compared with controls[80], while an EBMT[81] retrospective study, on much larger number of patients, has shown a clear advantage for ATG-treated patients on Alemtuzumab ones. A recent UK study[82] comparing the use of ATG with the use of Alemtuzumab for in vivo T-cell depletion (including HSCT from SIB, MUD and mismatched donors) showed that engraftment failure rates were inferior in the Alemtuzumab group when compared to the ATG group (9% vs 11%, respectively), with a similar survival outcome for sibling (91% vs 85%, respectively), and with a lower risk of chronic GvHD observed in the Alemtuzumab group (11% vs 26%). An additional way is to use "Alemtuzumab in the bag,"[83] producing ex vivo T-cell depletion: the method is probably not widespread, but may lead to significant protection against chronic and acute GvHD. Given this, the standard conditioning

regimen for patients with acquired AA undergoing an HLA-identical sibling transplant remains remain CY200 plus ATG or Alemtuzumab as T-depleting agents.

Independently from type of T-cell depletion, or presence of other HSCT outcome predictors, age at transplant contributes to the successful outcome and long term survival from HSCT. Older AA patients, with longer diagnosis-to-HSCT interval, and long history of previous IST and blood transfusions present a dismal outcome when compared with the youngest one[84]. The actual causes of treatment failures are not different from those seen in younger patients, including graft failure and mostly GvHD and its further complications (i.e., infections). In keeping with observations from HSCT in other settings (i.e., acute leukemias), different groups have investigated the possibility to reduce the intensity of the conditioning regimen, possibly sparing early toxicity which may trigger GvHD and other transplant-related complications. For this reason, Fludarabine (FLU) -based regimens have been also introduced in older adults[85]. In a retrospective EBMT study[86], patients older than 30 receiving FLU-based regimens were compared with a matched paired group of patients conditioned with Cy200-ATG over the same period of time (1998–2007): patients conditioned with FLU had a higher probability of OS than the control group, when adjusting for recipient's age, possibly related to a trend toward a reduced incidence of graft failure in patients receiving FLU (0% vs 11%), whereas no difference was observed regarding incidence of GvHD. In a more recent analysis[87] of the EBMT, the effect of age was combined with conditioning regimen in transplants from HLA-identical siblings, in the period 2001 through 2010 (See fig.6).

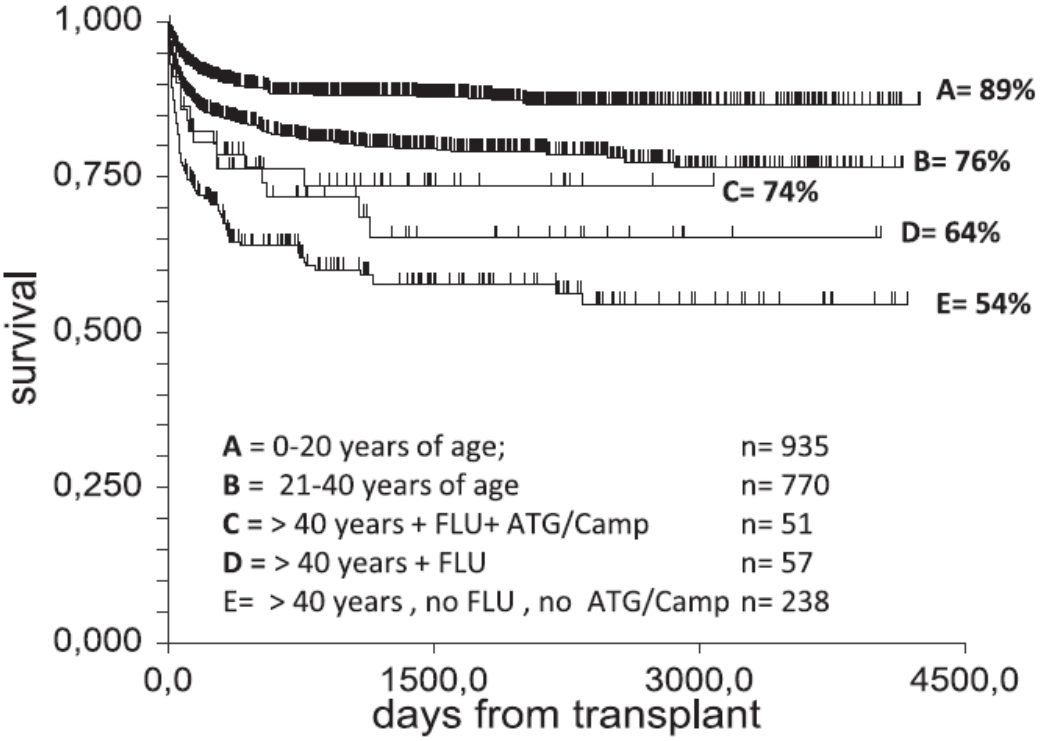

Fig.6. Effect of age combined with conditioning regimen in AA transplants from HLA-identical siblings (2001-2010).

Survival of patients aged 1 to 20 years was 89%; survival of those aged 21 to 40 was 76%; survival of patients older than 40 receiving a FLU-based regimen and ATG or Alemtuzumab in the conditioning regimen was 74%; survival of patients older than 40 receiving a FLU-based regimen, but no ATG and no Alemtuzumab, was 64%; and survival of those older than 40, not receiving a FLU-based regimen, and no ATG nor Alemtuzumab, was of 54%. This study suggests that survival can improve in patients older than 40 years if appropriate modifications are made in the conditioning regimen, using a combination of FLU and intermediate dose of Cy to reduce conditioning intensity. Finally, as for Cy200-ATG, the replacement of ATG with Alemtuzumab[88] seems to result in even better outcomes, eventually providing an additional conditioning regimen to be considered as standard of care. Determining the upper age limit for eligibility to transplant in AA remains a difficult issue. It would seem reasonable to restrict transplantation from identical siblings to AA patients who are younger than 60 years, using reduced intensity conditioning regimens, as described above.

Three different stem cell sources, namely bone marrow (BM), peripheral blood stem cells (PBSC), and granulocyte colony–stimulating factor (G-CSF)–mobilized BM (primed-BM), can be considered as graft source for HLA-matched transplantation from SIB donor. A first combined EBMT/Center for International Blood and Marrow Transplant Research (CIBMTR)[89] analysis has shown a significant survival advantage for BM grafts in young patients (<20 years) but not in older patients (≥ 20 years): this study also confirmed the increased risk of chronic GvHD for recipients of PBSC grafts. In 2012 the EBMT published a second analysis[90] on a much larger number of patients who received a first matched sibling transplant between 1999 and 2009, with BM or PB as a stem cell source. The conclusions of this study were unequivocal: PB grafts from HLA-identical siblings, when compared with BM grafts, were associated with a greater risk of acute grade II to IV GvHD (17% vs 11%) and a greater risk of cGvHD (22% vs 11). The survival disadvantage for PB in comparison with BM was significant in patients aged 1 to 19 years (76% vs 90%) and in those aged 20 years and older (64% vs 74%); this was true also for those older than 50 years (39% for PB vs 69% for BM). In an additional study[91] comparing BM, PB, and primed-BM, acute and chronic GvHD were again higher in PB graft recipients, and there was no advantage for G-CSF–mobilized BM in terms of engraftment and survival. Given this, BM should be the stem source of choice for HLA-identical stem cell transplants in patients with AA, while the use of PB should be discouraged due to association with worse survival and increased risk of GvHD, especially chronic.

Graft rejection, an historical problem in HSCT for AA, is now not frequent in patients who go directly to transplant, and with a modest transfusion burden, likely due to a beneficial loss of exposure to immunogenic products from fewer donors (leukocyte-depleted erythrocytes, platelet collected by cyto-apheresis). Conditioning regimens who do not include irradiation in sibling transplant regularly achieve sustained engraftment in more than 96% of cases treated with Cy200-ATG regimen. Similarly, FCA regimen has ensured acceptable engraftment rates in older patients with AA without irradiation. The use of chimerism[92] studies using short tandem repeat (STR) method, has been a major advance in our understanding of how donor/recipient myeloid and lymphoid cells interact. In a study[93] on 94 AA transplants, patients were classified as: 1) complete donor chimeras (43%), 2) transient mixed chimeras (16%), 3) stable mixed chimeras (20%), 4) progressive mixed chimeras (15%), and 5) early graft rejection (5%). This study showed that mixed chimerism occurs frequently in a large proportion of patients, especially early

after HSCT, and that kinetics of sequential chimerism is a predictor of outcome, with progressive mixed chimeras at higher risk of rejection or disease relapse. Treatment of mixed chimerism has not been standardized in AA. Mixed-chimerism associated with declining peripheral blood counts may be treated with low doses of donor lymphocyte infusions (DLI), while maintaining GvHD prophylaxis with cyclosporine. Unfortunately there are no reports on this approach, and the use of DLI for mixed chimerism continues on a patient-to-patient basis. Caution must be exercised with DLI dosing because of the potential risk of lethal GvHD in these patients.

HSCT from HLA-Matched Unrelated Donor. The outcome of MUD HSCT for patients with AA has constantly improved over the last 15 years. Better selection of HLA-matched donors, with introduction of high resolution HLA screening[94], and adoption of reduced intensity conditioning regimen, including FLU-based chemotherapy protocols and low-dose Total-Body Irradiation (2 Gy TBI) in adults[95], improved general overall survival in this setting, reaching currently reported level above 75%. Results of MUD HSCT have improved so much that now treatment guidelines[2] for AA consider it as a recommended second line treatment in adults failing to respond to one course of IST, while in children where a SIB donor is not available, a consistent growing amount of studies suggest that it may be considered even as first line treatment for severe and very severe AA.

Different centres match either for A, B, C and DRB1 at the allelic level, looking for 8/8 matched donors, or for DQ looking for a 10/10 match. In a recent EBMT analysis[96] was showed that an effect of mismatching (<8/8 vs 8/8 match) is evident only for patients prepared with FLU-CY and ATG (FCA), but not for patients receiving FCA supplemented with low-dose TBI (FCA-TBI). Therefore, an 8/8 (A, B, C and DRB1) matched donor would be ideal, but a 7/8 (or 9/10) match would probably be also acceptable and recommendable if low-dose TBI is added to conditioning regimen. The dose of CY for FCA was originally set at 300 mg/m^2. This was associated with a significant risk of rejection, so the current indication is to decrease the dose of CY to 120 mg/kg. Using the FCA regimen, EBMT study[96] showed that rate of acute GVHD grade II–IV was low (18%), with chronic GVHD more frequent, as expected, when TBI 2 Gy was added to overcame HLA-mismatching (50% vs 27%). This was particularly true when PB was used as a source of stem cells. In particular, risk of extensive chronic GVHD is 3% for BM, and of 20% for PB.

A study of the CIBMTR[97] has compared BM and PB in UD transplants for acquired AA: 296 patients received either BM or PB from unrelated donors, matched at HLA-A, -B, -C, and -DRB1 level. Hematopoietic recovery was similar after PB and BM transplantation. Acute grade II to IV GvHD was more frequent in PB than in BM (48% vs 31%). cGvHD risks were not significantly different after adjusting for age at transplantation. Mortality, independent of age, was higher after PB compared with BM transplantation (76% vs 61%). The investigators concluded that in UD transplants, similarly to HLA-identical siblings, BM is the preferred graft source in SAA. Finally, a general consensus exist on the concept that a marrow cell dose ranging between 3 to 4x10^8/kg seems to allow optimal outcome. Sib HSCT, Campath is emerging as an alternative option to ATG (FCC regimen)[88]. Using FCC, the risk of acute GVHD is 14% and chronic GVHD 4%, suggesting a potential low incidence of GVHD with this conditioning regimen.

Graft failure remain a clinical issue in MUD HSCT. Using the FCA[96] regimen, cumulative incidence of graft failure was 17% in the EBMT study. Patients with a longer interval from diagnosis to transplant (>2 years) had a trend for a higher risk of graft failure (22%) when compared with patients grafted <1 year (12%), or between 1

and 2 years from diagnosis (14%). Five-year survival were respectively of 87% and 55% for patients grafted within or beyond 2 years from diagnosis. Comparable graft failure rates were reported with FCC regimen even for the MUD HSCT setting.

Alternative donor HSCT for AA. The use of CBT in AA has not been systematically investigated. The largest study was reported by the EUROCORD[98] and EBMT, and included 71 patients grafted from either a single or double CB units. In this study, the outcome was not excellent (3-year survival 38%) mostly due to graft failure: indeed, neutrophil recovery at 2 months was achieved in 51% of patients only, and obviously associated with the number of nucleated cells contained in the CB unit. Sixty-eight percent of the patients received a Flu-based conditioning regimen; all the patients receiving a fully myeloablative conditioning regimen with TBI died. Similar outcomes were reported from the Japanese Registry; the possible beneficial effect of a reduced intensity conditioning regimen was also confirmed in a smaller series.

Haplo-identical HSCT remain an interesting option for AA, considering the fact that the great majority (if not all) patients should have some haplo-identical donors. Furthermore, the interest for this strategy has been revived by the recent seminal studies from the Johns Hopkins group in Baltimore, who successfully exploited post-transplant immunosuppression using high-dose CY. Historically, results with Haplo-identical HSCT has been disappointing; a recent EBMT[99] survey reported the outcome of 73 AA patients transplanted between 1976 and 2011, showed a 3-year overall survival of only 37%, largely as a result of lack of stable engraftment and/or development of GvHD or other transplant-associated complications. On the contrary, additional promising data were recently reported by the King's College group, who reported stable engraftment in six out eight AA patients grafted from a HLA haplo-identical donor using the Johns Hopkins protocol; notably, only one patient developed grade II acute GvHD, and no chronic GvHD was reported, irrespective of the fact that PBSC was used as stem cell source. Based on these data, CBT and Haplo-HSCT are actually not recommended for AA patients, and their use should be limited within prospective trials, or at least in few experienced transplant centers. It has to be remarked that in this specific HSCT setting, selection of an alternative donor should rule out the presence of HLA-antibodies specific for donor HLA antigens, given the highest risk of graft rejection highlighted.

4. AIMS OF THE STUDY

In 2011 Marsh et al[88] reported that a conditioning regimen including Fludarabine 30 mg/m^2 given daily x 4, Cyclophosphamide at doses of 300 mg/m^2 given daily x 4, and Alemtuzumab (the humanized anti-CD52 antibody Campath1-H), namely FCC, was associated with sustained engraftment and excellent outcome in AA HSCT both from SIB and MUD donors. Very interestingly, rate of engraftment was comparable with ones reported in literature, despite persistence of stable mixed chimerism in CD3+ T-cell fraction for a consistent percentage of patients. Most notably, rate of acute GVHD was of 13.5% (only grades I-II), and rate of chronic GVHD only of 4%. Finally, factors significantly associated with inferior outcome in HSCT for AA, like older age at transplant, longer interval from diagnosis to HSCT, and use of PBSC as a source of stem cell, did not impact significantly in this cohort. Presumably these clinical features, including low incidence of GVHD, were related to in vivo T-cell depletion with Alemtuzumab, and the establishment of mixed donor/host chimerism in the CD3+ T-cell compartment, suggesting that FCC may work as an "immune-ablative" conditioning regimen in adults with AA.

Nevertheless, some criticism in this study were present. Firstly, data collected were mainly retrospective, with an heterogeneity in patient cohort derived from the multicenter nature of the study, with different dosage of Alemtuzumab used per patient. Secondly, patients follow-up was relatively short, with a median time of observation reported of 18 months, that remained quite unsatisfactory in providing data on long term survival and the intriguing topic of the persistent CD3+ mixed chimerism.

Finally, even if the same authors defined FCC as an immune-ablative conditioning regimen, no data were provided about immune reconstitution after this type of HSCT, with eventual correlation with excellent outcome in AA.

Given this, aims of this study were:

- ✓ To confirm excellent clinical outcome of FCC HSCT for AA patients, particularly in terms of graft failure and GvHD incidence, and long term survival.

- ✓ To analyze immune reconstitution in this specific setting of patients, providing correlation with clinical outcome.

- ✓ To analyze long term chimerism trend and impact of persistent mixed chimera on immune phenomena after HSCT, especially graft failure and GvHD.

5. PATIENTS, MATHERIALS AND METHODS

5.1 Study population

All patient receiving HSCT for AA at King's College Hospital from January 2007 to June 2015 were given access to the study. During this period, 45 patients were enrolled (Male/Female, 28/17). Median age at transplantation was of 32 years (Range 15 - 63), with 14 (31.1%) patients older than 50 years. AA was idiopathic in 91.2% of cases. HSCT was performed after immunosuppressive therapy in 33 (73.3%) patients, with ATG + CyA as preferred regimen. At diagnosis, a PNH clone was detected by flow cytometry in 21 (46.6%) patients. Baseline patients characteristics before HSCT are summarized in table 2.

According to Marsh et al, FCC conditioning regimen was administered as follow: Cyclophosphamide 300 mg/m^2 IV daily x 4 days, given from day −7 to −4; Fludarabine 30 mg/m^2 IV daily x 4 days, given from day −7 to −4; Alemtuzumab was given 0,2 mg/Kg IV or SC daily x 5 days, from days −7 to −3. Stem cells were given at day 0, both from BM or PBSC source, according to investigator choice and donor availability. TBI (total dosage of 2 Gy) was added to conditioning protocol in 7/8 HLA mismatched MUD transplant to improve engraftment according with previous observations.

Post-HSCT GvHD prophylaxis consisted in CyA (starting dosage 2.5 mg/kg IV) from day −1 on, and titrated to plasma trough levels of 200-300 ng/mL. Oral CyA was substituted when a good oral intake was achieved, and continued for 12 months, with tapering at 9 months in presence of stable hematologic parameters and without declining mixed donor chimerism, and complete suspension at one year after HSCT, in absence of any sign of GvHD.

Supportive care with irradiated blood products and antimicrobial treatment were provided according to institution practices and EBMT recommendations for AA patients. All patients were screened for CMV and EBV infections/reactivation at least weekly for the first 3-6 months using PCR-based assay.

Neutrophil engraftment was defined as the first of 3 consecutive days with an absolute neutrophil count (ANC) ≥0.5 x 10^9/mmc, unsupported by G-CSF. Platelet engraftment was defined as the first of 3 consecutive days with a platelet count ≥ 20x10^9/mmc, without platelet transfusion support for 7 preceding days. Patients were considered evaluable for engraftment if they survived more than 21 days after transplantation. Primary graft failure was defined as the absence of neutrophil count ≥0.5x10^9/mmc on 3 consecutive days, and late graft failure as recovery followed by recurrent pancytopenia with a hypocellular BM in the absence of severe GVHD. Acute and chronic GvHD was diagnosed on clinical grounds with histopathologic biopsy where possible and graded according to published NIH criteria.

Study protocol was approved by King's College internal review board and Ethical Committee, and was conducted in accordance with the Declaration of Helsinki. All patients signed separated informed consent form for inclusion in clinical treatment group, and for collection of biological samples.

Table 2. Patients characteristics, FCC cohort	
Number of patients	45
Median age (range)	32 (15 – 63) years
Number of patients aged > 50yr	14 (31.1%)
M : F	28 : 17
Aetiology of aplasia	
- **Idiopathic**	41 (91.2%)
- **Post-hepatitic (seronegative)**	2 (4.4%)
- **Eosinophilic fasciitis**	1 (2.2%)
- **Coeliac disease**	1 (2.2%)
IST previous HSCT	
- **Matched sibling (MSD)**	6 of 12 (50%)
- **Unrelated donor (UD)**	27 of 33 (81.8%)
Number of patients HLA alloimmunised	11 (24.4%)
Number of patients with PNH clone	21 (46.6%)
Median PNH clone size:	
- **granulocytes**	2% (range 0.02 – 40%)
- **monocytes**	2.85% (range 0.01 – 32%)

5.2 Immunophenotypic analysis

PB samples for immune reconstitution analysis were collected from patients at day +30, +60, +90, +180 and + 360 after HSCT. Peripheral Blood Mononuclear Cells (PBMC) were separated by a gradient centrifugation with Ficoll-Paque Plus system (GE Healthcare, Sweden), and aliquots were stored frozen in liquid nitrogen after DMSO resuspension to preserve viability. When prepared for Immunophenotypic analysis, PBMCs were thawed rapidly in 37^{o}C water bath, transferred to RPMI1640 colture medium with 10% fetal calf serum (FCS) and 1% Penicyllin/Streptomycin (P/S), and then centrifuged at 1300 rpm (300g) for 10 minutes at room temperature, to remove DMSO. Cell viability was subsequently assessed by cell counting in Neubauer chamber after L/D staining with trypan blue. A minimal amount of 0.5 to 1x10^{6} cell, with viability >90%, was accepted to proceed for staining with specific antibody panel. After cell counting, two additional washing step were performed with resuspension in PBS only (in the absence of any protein) and centrifuge at 1300 rpm (300g) for 10 minutes at room temperature.

An appropriate volume of fluorochrome-labeled monoclonal antibodies cocktail was then added to relevant sample, according to specific lymphocyte subset, for surface staining. An intracellular Foxp3 staining step was additionally performed after permeabilization (with BD Biosciences Cytofix/Cytoperm solution), according to the manufacturer's instructions, when needed.

Data on fluorochrome-coniugated antibodies, including clone type, fluorochrome, supplier and amount used according to manufacturer's instructions are listed in table below (Table 3 a,b,c,d):

Panel	Parameter	Detector	µL per 0.5x10^6 cells	Supplier	Clone
NK, B, γδ T-cells	CD3 VioGreen	V525	1	Miltenyi Biotech	BW264/56
	CD56 PerCP-Cy5.5	B710	2.5	BD Biosciences	B159
	CD16 eFluor 450	V450	2	eBioscience	CB16
	CD19 APC	R670	10	BD Biosciences	HIB19
	Pan gd TCR PE	YG586	2	eBioscience	B1.1
	TCR V g9d2 FITC	B530	1	Beckman Coulter	No carry
	L/D eFluor 780	R780 dump channel for Monocyte and L/D	1	eBioscience	No carry
	CD14 APC- eF 780		2.5	eBioscience	61D3
	CD45 AlexaFluor 700	R730	1	Biolegend	HI30

Table 3a. Staining cocktail for characterization of NK, B-cells and γδ T-cells.

Panel	Parameter	Detector	µL per 0.5x10^6 cells	Supplier	Clone
T-cell subsets	CD3 VioGreen	V525	1	Miltenyi Biotech	BW264/56
	CD4 PerCP Cy5.5	B710	2.5	eBioscience	RPA-T4
	CD8 eFluor 450	V450	2.5	eBioscience	RPA-T8
	CD45RA APC	R670	10	BD Bioscience	UCHL1
	CD27 FITC	B530	2.5	eBioscience	323
	CD62L PE	YG586	10	BD Biosciences	DREG-56
	CD31 PE-Cy7	YG780	2.5	Biolegend	WM59
	CD14 APC-eFluor 780	R780 dump channel	2.5	eBioscience	61D3
	L/D eFluor 780		1 (all cells)	eBioscience	No carry
	CD45 Alexa Fluor 700	R730	1	Biolegend	HI30

Table 3b. Staining cocktail for characterization of T-lymphocytes (T-cells) subsets in PBMCs.

Panel	Parameter	Detector	μL per 0.5x10^6 cells	Supplier	Clone
T-regs	CD3 VioGreen	V525	1	Miltenyi Biotech	BW264/56
	CD4 eFluor 450	V450	1	eBioscience	RPA-T4
	CD27 FITC	B530	2.5	eBioscience	323
	CD25 APC	R670	5	BD Bioscience	BC96
	CD45RA PE	YG586	10	BD Bioscience	HI100
	CD127 PE-Cy7	YG780	2.5	eBioscience	RDR5
	Foxp3 PerCP Cy5.5	B710	1.5	eBioscience	PCH101
	L/D eFluor 780	R780	1 (all cells)	eBioscience	No carry

Table 3c. Staining cocktail for characterization of T-regulatory cells (T-regs).

Panel	Parameter	Detector	μL per 0.5x10^6 cells	Supplier	Clone
B-cells	CD19 APC	R670	10	BD Bioscience	HIB19
	CD27 PerCP-Cy5.5	B710	2.5	BD Bioscience	MT271
	CD38 FITC	B530	10	BD Bioscience	HIT2
	CD24 PE	YG586	10	BD Bioscience	ML5
	IgM Pacific Blue	V450	5	Biolegend	G20-127
	IgD APC-H7	R780	2.5	BD Bioscience	IA6-2

Table 3d. Staining cocktail for characterization of B lymphocytes (B-cells).

Nine-color analysis was performed by flow cytometry using a BD LSR-Fortessa FACS cell analyzer (BD Biosciences). A minimum of $1x10^5$ events were acquired per sample; acquired results were analyzed with FlowJo software (TreeStar). Lymphocytes subset were defined as per recently updated Human Immunophenotyping Consortium reported by Finak et al. (see table 4). Lymphocytes numbers were calculated from percentage values, based on an absolute lymphocyte count of the blood sample obtained using an automated leukocyte counter.

Lymphocytes Subset	Phenotype
B-cell	CD45+ CD19+ CD3-
Transitional (T1&T2)	CD19+ CD3- CD24-bright CD38-bright IgM-bright IgD-bright
Naive	CD19+ CD3- CD24-int CD38-int IgM-int IgD-int
Memory	CD19+ CD3- CD24-bright CD38-dim IgM-int IgD-dim
NK-cell	CD45+ CD3- CD56+ CD16+ or CD3- CD56-dim CD16+
Gamma/delta T-cells	CD45+ CD3+ pan gdTCR+
Vg9d2 T-cells	CD45+ CD3+ pan gdTCR+ Vg9d2 TCR+
CD4+ T-cell	CD3+ CD8- CD4+
CD4+ Naïve	CD3+ CD4+ CD45RA+ (or CD45RO-) CD27+
CD4+ Memory	CD3+ CD4+ CD45RA- (or CD45RO+) CD27+
CD4+ Effectors	CD3+ CD4+ CD45RA- (or CD45RO+) CD27-
CD4+ Terminal effectors	CD3+ CD4+ CD45RA+ (or CD45RO-) CD27-
CD4+ Recently Thymic Emigrants	CD3+ CD4+ CD45RA+ CD31+ CD62L+
CD4+ Regulatory T-cell	CD3+ CD4+ CD25-high CD27+ CD127-low Foxp3+
CD8+ T-cell	CD3+ CD4- CD8+
CD8+ Naïve	CD3+ CD4- CD8+ CD45RA+ (or CD45RO-) CD27+
CD8+ Memory	CD3+ CD4- CD8+ CD45RA- (or CD45RO+) CD27+
CD8+ Effectors	CD3+ CD4- CD8+ CD45RA- (or CD45RO+) CD27-
CD8+ Terminal effectors	CD3+ CD4- CD8+ CD45RA+ (or CD45RO-) CD27-

Table 4. Lymphocytes subset defined as Human Immunophenotyping Consortium (2016).

5.3 Functional B-cell assay for IL10-production

For characterization of IL10 B-cell production, PBMCs were co-coltured with irradiated CD40L+ L-cells for 12 hours (Ratio 1:10). Phorbol myristate acetate (PMA) (50 ng/mL), Ionomycin (250 ng/mL) (Sigma-Aldrich), and Brefeldin A (5 µg/mL, GolgiPlug; Sigma-Aldrich) were added for the last 6 hours of the culture. Cells were harvested, washed in staining buffer, and incubated as previously described with CD19-APC, CD38-FITC and CD24 PE (BD Bioscience) for surface staining. was

obtained. IL-10 production was assessed on gated $CD19^+$ B cells by intracellular cytokine assay. Cells were fixed/permeabilized (eBioscience) and stained with PerCP-Cy5.5-conjugated IL-10. At this stage, 20 µL of FcR blocking reagent (Miltenyi Biotec) was added. All data were acquired using BD-FACSCalibur (Becton Dickinson) and analyzed with FlowJo software.

CD40L-transfected liver mouse fibroblasts (CD40L-cells) and non-transfected control fibroblasts (L cells) (kindly provided by Dr L Barber, King's College, London, UK) were grown in RPMI 1640 with 10% FCS.

5.4 CMV tetramer staining

Tetramer staining and peptide library functional assay were used to identify CMV specific T-lymphocytes. Pro5®, a PE fluorochrome-conjugated MHC class I pentamer (Proimmune, Oxford, UK), containing an HLA-A*0201-restricted NLV peptide (NLVPMVATV - pp65, 495–503) was used for identification of CMV peptide-specific CD8+ T-cells. PBMCs were stained with appropriate pentamer amount (15 min at ambient temperature, 10 µl per 1-2x10^6 cells), washed and then incubated (30 min, 4°) together with anti-CD8 PerCP-Cy5.5 and anti-CD4 APC antibodies, in order to identify specific T-cell subpopulations.

5.5 Chimerism analysis

Samples (PB and BM) for chimerism study were collected from patients after HSCT at day +30, +60, +90, +180, and then annually. Chimerism was assessed in isolated peripheral blood $CD3^+$ T-cell and CD15+ granulocyte populations, as well as unfractionated bone marrow samples. Density gradient centrifugation was employed to separate low and high-density cells from whole peripheral blood samples (Density 1·077 g/ml) (Histopaque; Sigma Diagnostics, St Louis, MO, USA). An AutoMACS magnetic cell separator (Miltenyi Biotec Ltd, Surrey, UK) was used to isolate the cells (>95% purity) from peripheral blood samples and DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen Ltd, Crawley, UK). Fifteen polymorphic short tandem repeat (STR) loci (Penta E, D18S51, D21S11, TH01, D3S1358, FGA, TPOX, D8S1179, vWA, Penta D, CSF1PO, D16S539, D7S820, D13S317 and D5S818) plus the sex-determining Amelogenin loci (PowerPlex®; Promega Corp, Madison, WI, USA) were amplified to provide a genetic profile for pre-transplant donor and recipient samples. Informative alleles were identified and used to assess donor engraftment. The samples were electrophoresed using polyacrylamide gel electrophoresis on an ABI Prism 377 DNA sequencer and analysed using Genescan 2·0 software (Applied Biosystems, Foster City, CA, USA).

Quantification was carried out using the area under the peaks; cell dilution studies found the sensitivity of this methodology to be approximately 5%. Therefore, full donor chimerism (FDC) was defined as the presence of >95% donor haematopoietic cells, while MDC was defined as presence of 5–95% donor cells post-HSCT. Patients who showed mixed donor-recipient chimerism, but who subsequently reverted to full donor chimerism were classified as having transient mixed chimerism. Declining donor chimerism was defined as a 10% or greater increase in recipient chimerism on 2 consecutive measurements.

5.6 Cell sorting for chimerism analysis of specific cell subsets

PBMCs from frozen aliquots were labeled as previously described, with a 6 colours staining cocktail including CD56, CD19, CD3, CD4, CD45RA, CD27 in order to separate NK-cells, B-cells, CD4+ and CD8+ T-cell subsets (Naïve, memory and effectors). Cell concentration achieved before sorting procedure was about 5 to 15 $x10^6$ cells/ml, and addition of 1mM Na_2EDTA to PBS resuspended cells was used to prevent cell clotting. Finally, PBMCs were purified with by high-throughput fluorescent-activated cell sorting with BD FACSAria II sorter. DNA extraction and STR analysis for chimerism assessment was made according to aforementioned procedure above.

5.7 Statistical analysis

Time-to-event outcomes were computed by using the Kaplan-Meier technique and were compared by using the two-tailed log-rank test. Overall survival (OS) was measured from day 0 to death from any cause or last known follow-up. Event-free survival (EFS) was defined as survival with sustained engraftment, with death and graft failure categorized as treatment failure. EFS was measured from day 0 to the first indicator of graft failure, death of any cause, or last known follow-up. Cumulative incidence of events (graft failure, GvHD) were estimated by using competing risk methods. Impact of specific variable on time-to-event outcomes was assessed by univariate and multivariate comparison analysis with Cox proportional hazards regression model. All data were censored as of the June 1, 2015.

Differences in specific groups characteristics were analyzed using chi-square with Fisher's exact correction as appropriate.

Statistical comparisons between biological variables were performed using the non-parametric Mann-Whitney U-test and Kruskal-Wasllis method. Differences were considered statistically significant when *p* values were less than 0.05.

Statistical analysis was performed using SPSS 19.0 for Windows software (SPSS Inc.) and GraphPad Prism version 6.00 for Windows (La Jolla, California USA).

6. RESULTS

6.1 Patient characteristics, engraftment and chimerism trend

HSCT from a Matched Sibling donor (MSD) was performed in 12 (26.6%) patients, and in 33 (73.4%) from an Unrelated Donor (UD), 8 (24.2%) of whom received 2 Gy TBI for 9/10 mismatch. Graft source used was PBSC in 38 (84.5%) patients, and BM in 7 (15.5%). The median interval from diagnosis to HSCT was of 5.4 months (Range 2,5 – 34,7). The median CD34+ cell dose given was of 6.55 (Range 1.97 - 12.4)x10^6/Kg. Engraftment rate was excellent, with a median time to neutrophil recovery of 11 days (Range 10 - 22), and to platelet recovery of 12 days (range 9 - 61). Only one (2,2%) primary graft failure was recorded after transplant, with a second HSCT successfully performed as salvage treatment. No episodes of late onset graft failure/rejection were recorded in this cohort of patients (Table 5).

Chimerism status data were sequentially available in 42 (93.3%) patients for unfractioned BM, CD3+ and CD15+ PB cells (respectively lymphoid and granulocytic chimerism). Pattern of persistently mixed lymphoid chimerism, as previously observed with FCC conditioning regimen, was confirmed in this cohort of patients; median level of CD3+ chimerism was respectively 52.7% (SE ±7.1%), 61.7 (SE ±5.7%) and 81.8 (SE ±4.9%) at day +90, at +1 year and at +3 year after transplantation, even after CyA suspension. On the contrary, rapid and persistent full donor engraftment for PB granulocytic fraction and Unfractioned BM was promptly observed in all but one patient at day +90 onward. Very interestingly, higher level of CD3+ chimerism, with achievement of persistent full donor engraftment, both for lymphoid and granulocytic fraction, was observed in 8 patients (17.3%), 6 of them receiving TBI as part of their conditioning regimen (Fig. 7).

6.2 GvHD, infections and autoimmunity

Acute GvHD was observed in 6 (13.3%) patients, all of grade I-II. Chronic GvHD was observed in 5 (11.1%) patients, (3 mild, 1 moderate, 1 severe as per NIH scoring system), all with skin as the only site of involvement. Cumulative incidence of GvHD at 1 year was respectively of 16,2% (Fig.8). On uni- and multivariate analysis with Cox regression model, factors significantly predicting chronic gvhd were 1) previously diagnosed acute GVHD (p=0.035) and 2) CD3+ chimerism at day+100≥90% (p=0.006). GvHD contributed to the death of only one patient, where severe skin involvement was associated with occurrence of multiple thrombosis and septicemia.

EBV viremia was detectable in 20 (44.4%) patients, but for only two (4.4%) of them a specific treatment was needed: in one patient a proper diagnosis of post-transplant lymphoproliferative disorder (PTLD) was made, while in the other one therapy with rituximab was started only for excessive viral load, but in absence of signs of lymphoproliferative disease. CMV viremia was detected in 11 (24.2%) patients, but no one progressed to CMV disease due to pre-emptive therapy. Adenovirus viremia was observed in 3 (6.6%) patients. Invasive Fungal infections (IFI) were diagnosed in three (6.6%) patients, with fatal outcome for two of them.

Seven cases (15.5%) of post-HSCT autoimmune disorders were recorded. Immune-mediated cytopenia were observed in 6 patients (13.3%) (2 PRCA, 3 Haemolytic Anemia, 1 Autoimmune neutropenia), while in 1 (2.2%) an autoimmune hypothyroidism with detectable auto-antibodies was diagnosed.

Table 5. post-HSCT clinical data	
Type of donor - **Matched sibling (MSD)** - **Unrelated donor (UD)** - **9/10 UD**	 12 (26.6%) 33 (73.4%) 8 of 33 (24.2% of UD)
Median time to transplant - **MSD** - **UD**	 5.4 months (Range 2.7 – 34.7) 8.4 months (Range 2.1 – 178.9)
Stem cell source - **BM** - **PB**	 7 (15.5%) 38 (84.5%)
Median Alemtuzumab dose	70 mg (Range 45 – 100)
Median CD34+/Kg stem cell dose	$6.55x10^6$ (Range 1.97 – 12.40)
Median follow-up	31.4 months (Range 3 – 93)
Median time to ANC > 0.5 x 10^9/l	12 days (Range 10 – 22)
Median time to platelets > 20 x 10^9/l	12 days (Range 9 – 61)
Primary graft failure	1 (2.2%)
1 yr TRM	3 (6.6%)
Acute GVHD: - **Grade I/II** - **Grade III/IV**	6 (13.3%) - 6 of 6 (100%) - 0 of 6
Chronic GVHD: - **Mild** - **Moderate** - **Severe**	6 (13.3%) - 4 of 6 (66%) - 1 of 6 (17%) - 1 of 6 (17%)
Documented viral infections: - **Adenovirus** - **CMV** - **EBV**	34 (75.2%) - 3 (6.6%) - 11 (24.2%) - 20 (44.4%)
Post-HSCT autoimmunity: - **Immune cytopenia** - **Other**	7 (15.5%) - 6 (13.3%) - 1 (2.2%)

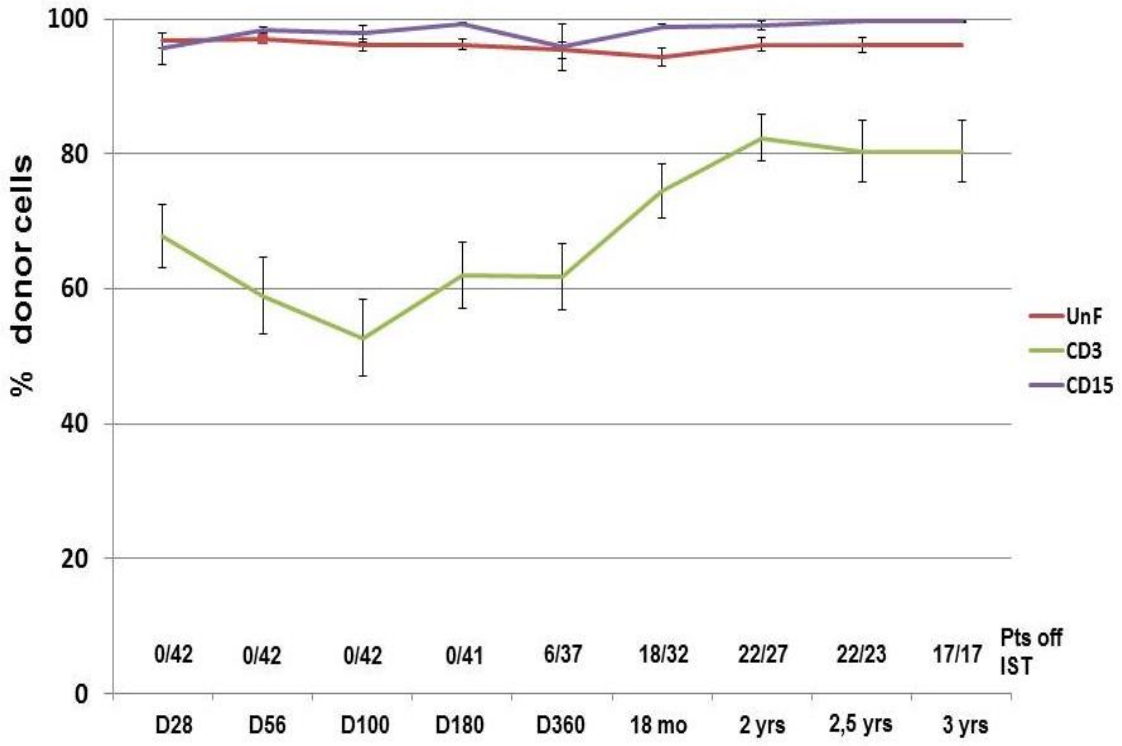

Fig.7. Post-HSCT chimerism trend. Unf, unfractioned bone marrow; CD3, lymphoid fraction; CD15, granulocytic fraction.

6.3 Survival analysis

Overall survival and event-free survival were respectively of 93.1% and 90.7% at 1 years after transplant, without further decrease on prolonged follow-up (Fig.8). Three patients died after HSCT, accounting for an overall TRM rate at 1 year of 6.6%. Cause of death were IFI for two of them, and multi-organ failure due to haemolytic anemia, septicemia and severe chronic GvHD in one of them.

No significant impact of age, stem cell dose, stem cell source and type of donor was observed on Cox Univariate and Multivariate regression analysis, both for OS (Fig.9) and EFS (Fig.10) in this cohort of patients.

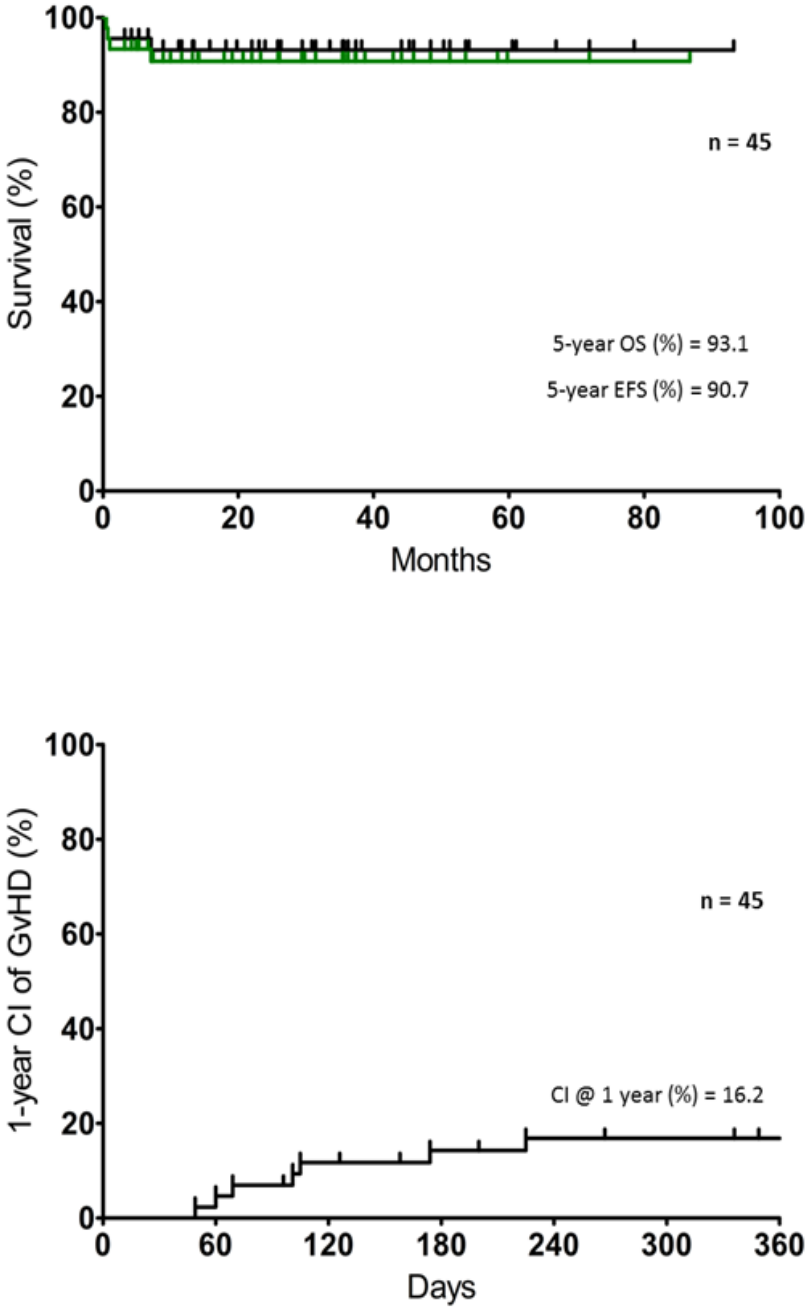

Fig.8. On top, General Overall and Event-free Surival. Below, Cumulative Incidence of GvHD at 1 year.

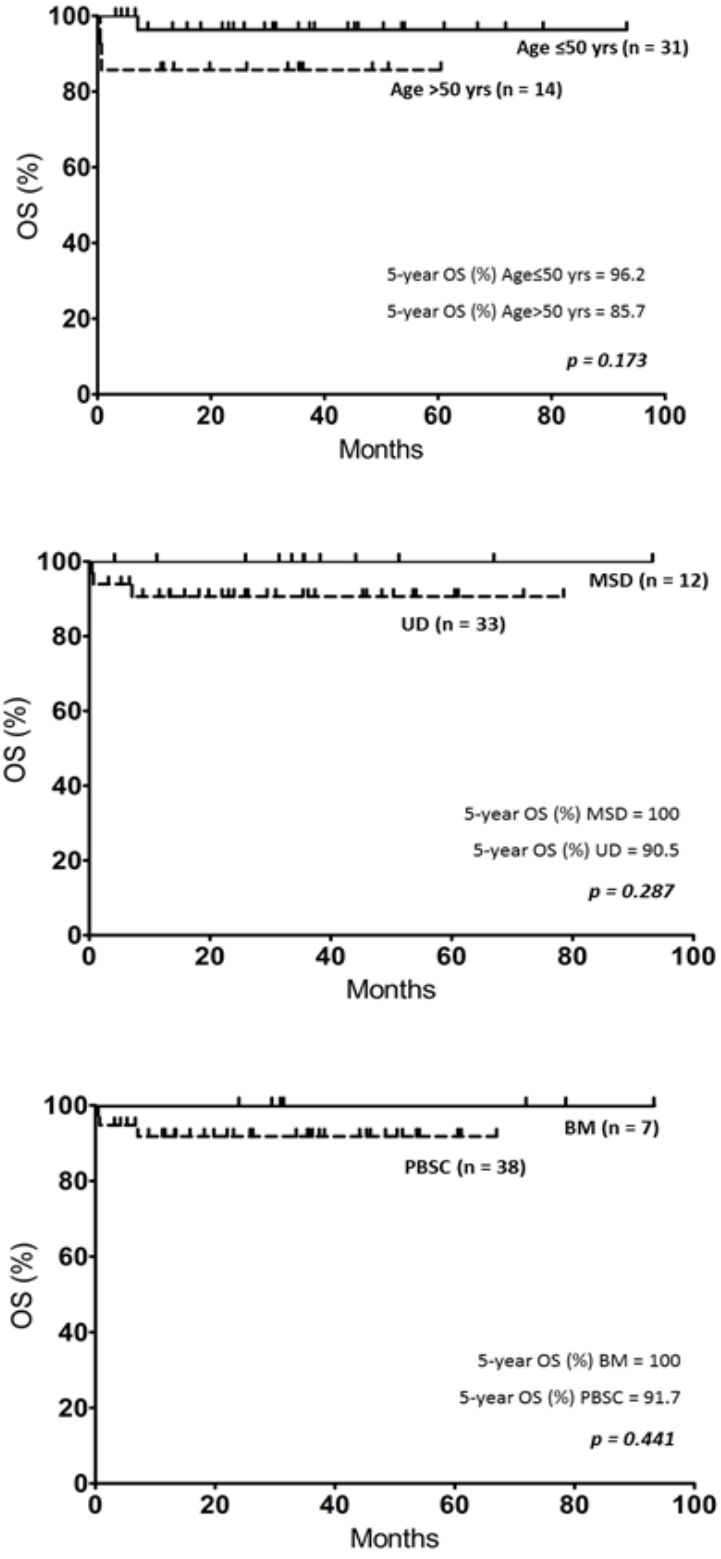

Fig.9. No significant impact of Age, Donor, Stem cell source on OS.

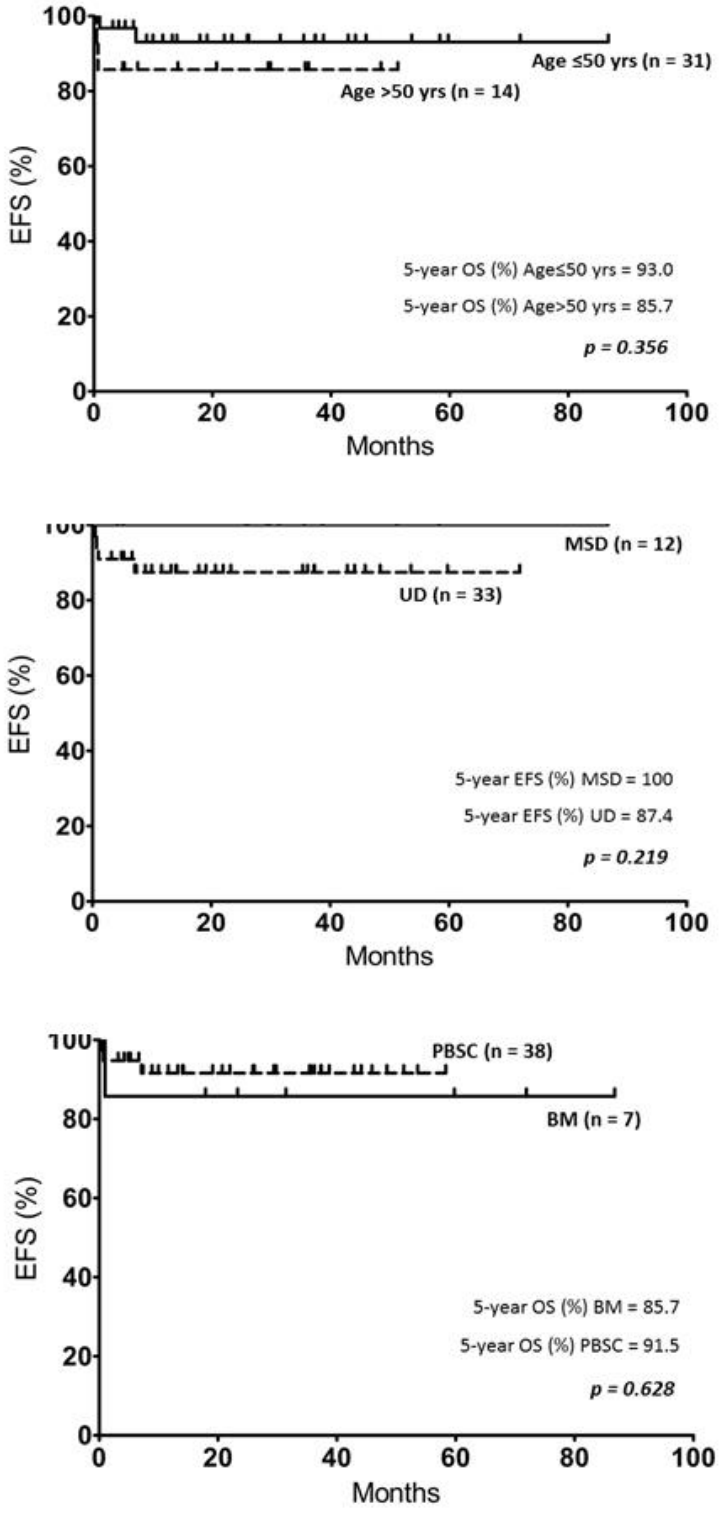

Fig.10. No significant impact of Age, Donor, Stem cell source on EFS.

6.4 Immune reconstitution dynamics after HSCT

As expected from an Alemtuzumab based regimen, a profound depletion of all lymphocyte subsets is observed after transplant, with level of total lymphocytes remaining significantly lower ($P<0,005$) than normal still one year after transplantation. Sequential analysis of peripheral blood lymphocytes composition to monitor immune reconstitution showed that B-lymphocytes and NK-cells recovered first, with NK cells being the main lymphocyte population at day 90, accounting for a median level of 31,7% ($p<0,005$) and 28,4% ($p<0,005$) when compared to level of healthy age-matched volunteers. However B-cell recovery was faster and more robust than NK-cells, with numbers of B-cells reaching the normal range at day 90 along with $\gamma\delta$T-cell after transplantation (Data shown in figure 11 and 12).

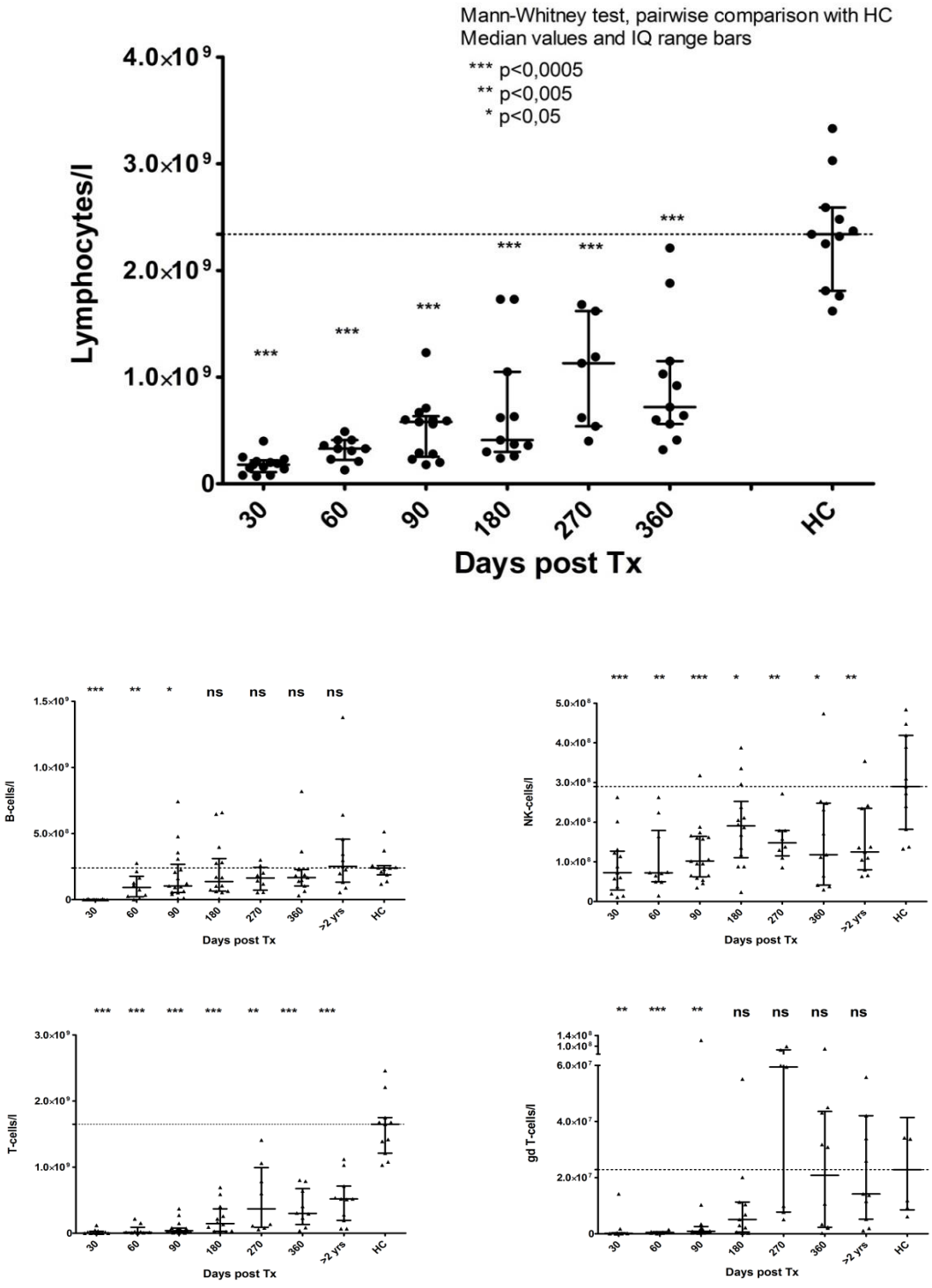

Fig. 11. Total lymphocytes, with B-cell, NK-cell, T-cell and γδT-cell frequencies (n° of cell/mmc) after FCC HSCT. As expected from an Alemtuzumab based regimen, cell counts remain extremely low, with only B-cells and gdT-cell recovering at almost normal level within 3 months after transplant. Frequencies compared with healthy control group by Mann-Whitney U-test.

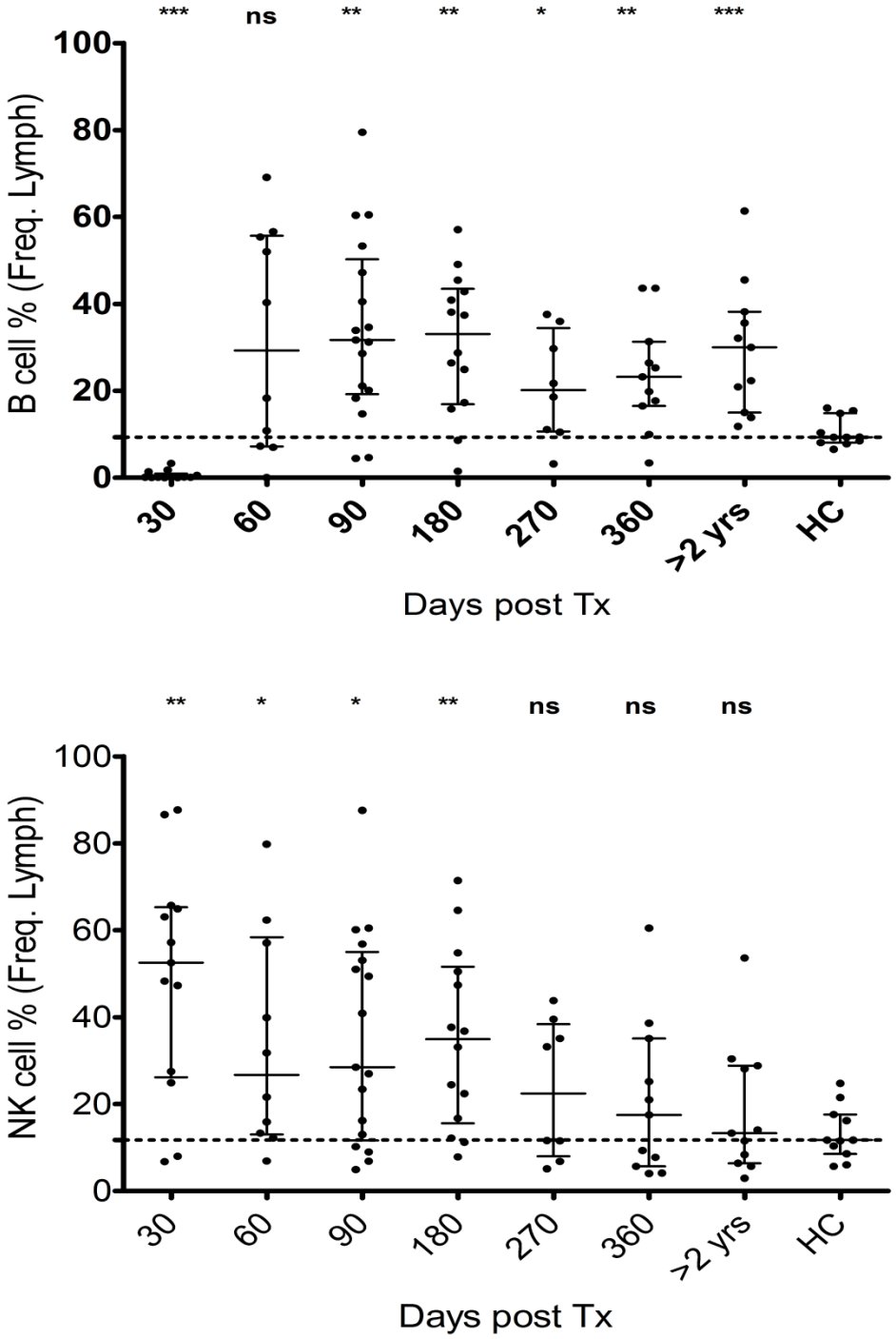

Fig. 12. Proportion (% of lymphocytes/mmc) of B-cell and NK-cell after FCC HSCT. Proportions of B and NK-cells remain significantly over normal within first six months after HSCT. Proportion compared with healthy control group by Mann-Whitney U-test.
*** = p<0,05 ; ** = p<0,005; *** = p <0,0005; NS = p>0,05**

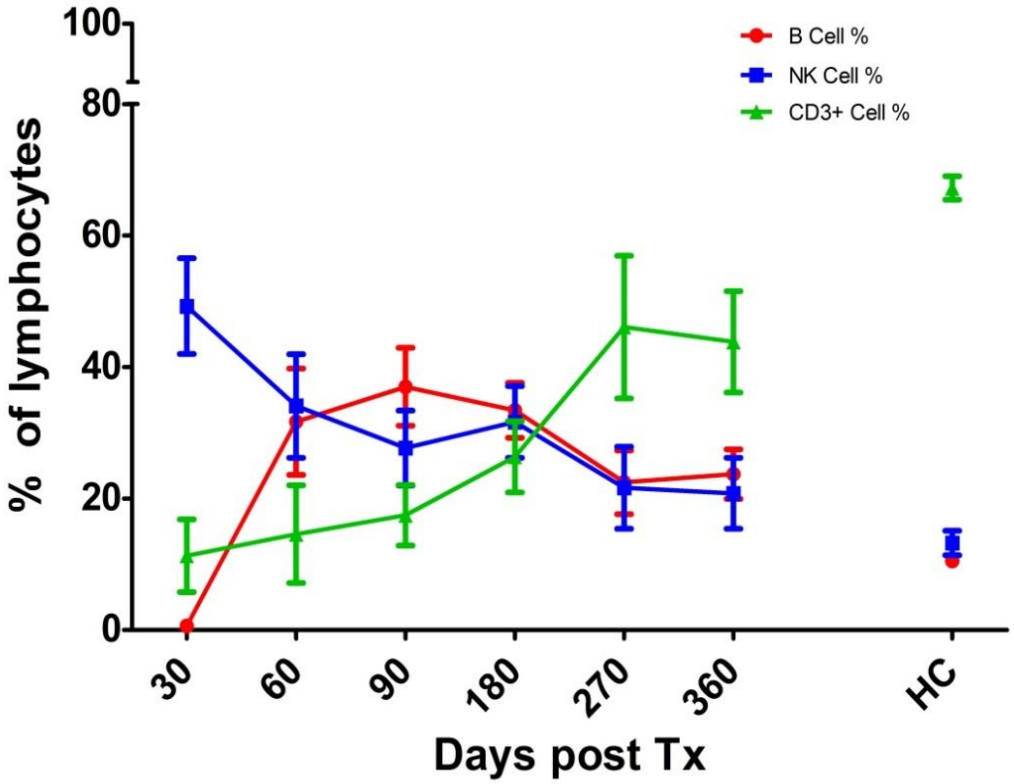

Fig. 13. Relative proportion (% of lymphocytes/mmc) of T-cell, B-cell and NK-cell after FCC HSCT. B-cell and NK cell remain the predominant lymphocyte type until one year after FCC HSCT, when proportion of T-cell tend to increase.

CD3+ T-cells remained profoundly deficient after transplant, comprising only 1.76% of total lymphocytes at day 30, and rising up to 43.8% by day 360, but still significantly below normal (66%, p=0.018) (Fig. 13).

T-cell subset composition was investigated by analyzing expression of CD4+ and CD8+ surface antigen to discriminate between Helper and cytotoxic lymphocytes. Investigation of T-cell subsets was made by surface marker expression analysis of CD45RA, which expressed on naïve cells, and co-stimulatory molecule CD27, which is lost during antigen-induced differentiation to effector elements.

Number of CD4+ T-cells and CD8+ T-cells remained significantly below normal within 1-year after HSCT, but in terms of recovery CD8+ were proportionally faster than CD4+ T-cells, producing as consequence a disruption with inversion of physiological CD4+/CD8+ T-cell ratio observed in peripheral blood as for healthy individuals (Figure 14).

Cell numbers, expressed as n° of elements/mmc, within CD4+ and C8+ T-cell subsets remained profoundly deficient after HSCT. However some differences were present: composition of both CD4+ and CD8+ T-cell populations was relatively defective in naïve cells and memory cells, especially when compared to age–matched healthy volunteers. On the other hand, number of terminally differentiated effector elements (Effectors, terminal effectors or total effectors) tended to recover within normality at 1-year after HSCT in the CD8+ T-cell fraction (data shown in fig.15 and fig. 16).

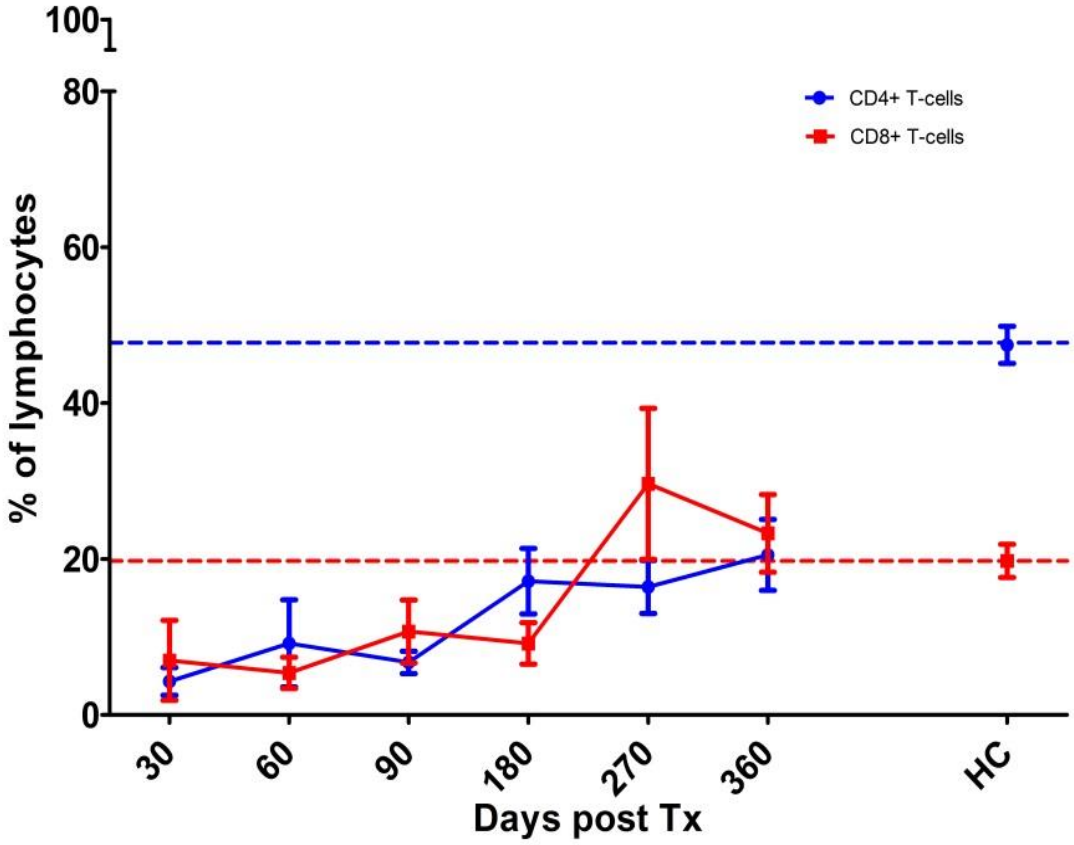

Fig. 14. Relative recovery (% of lymphocytes/mmc) of CD4+ and CD8+ T-cells after FCC HSCT. Disruption of normal CD4+/CD8+ ratio observed in comparison with healthy individuals.

When immune reconstitution of T-cell was analyzed in term of percentage of T-lymphocytes, within the CD4+ T-cells subset the proportion of Naïve cells increased constantly after HSCT, reaching a level at day 360 proportionate to healthy control group (p=0,131), and reconstituting an almost normal subset composition with Memory and Effectors at the same time-point after transplantation (Figure 17).

Moreover, expression of surface markers associated with recent thymic egression (RTEs = $CD31^+$ $CD62L^+$ $CD45RA^+$) that indicates renewed thympoiesis was evaluated within CD4+ T-cell Naïve cells. Patients aged less than 50 years showed more effective recovery of RTEs, indicating renewed thymopoiesis, and potential implementation of central tolerance after transplant (Figure 18).

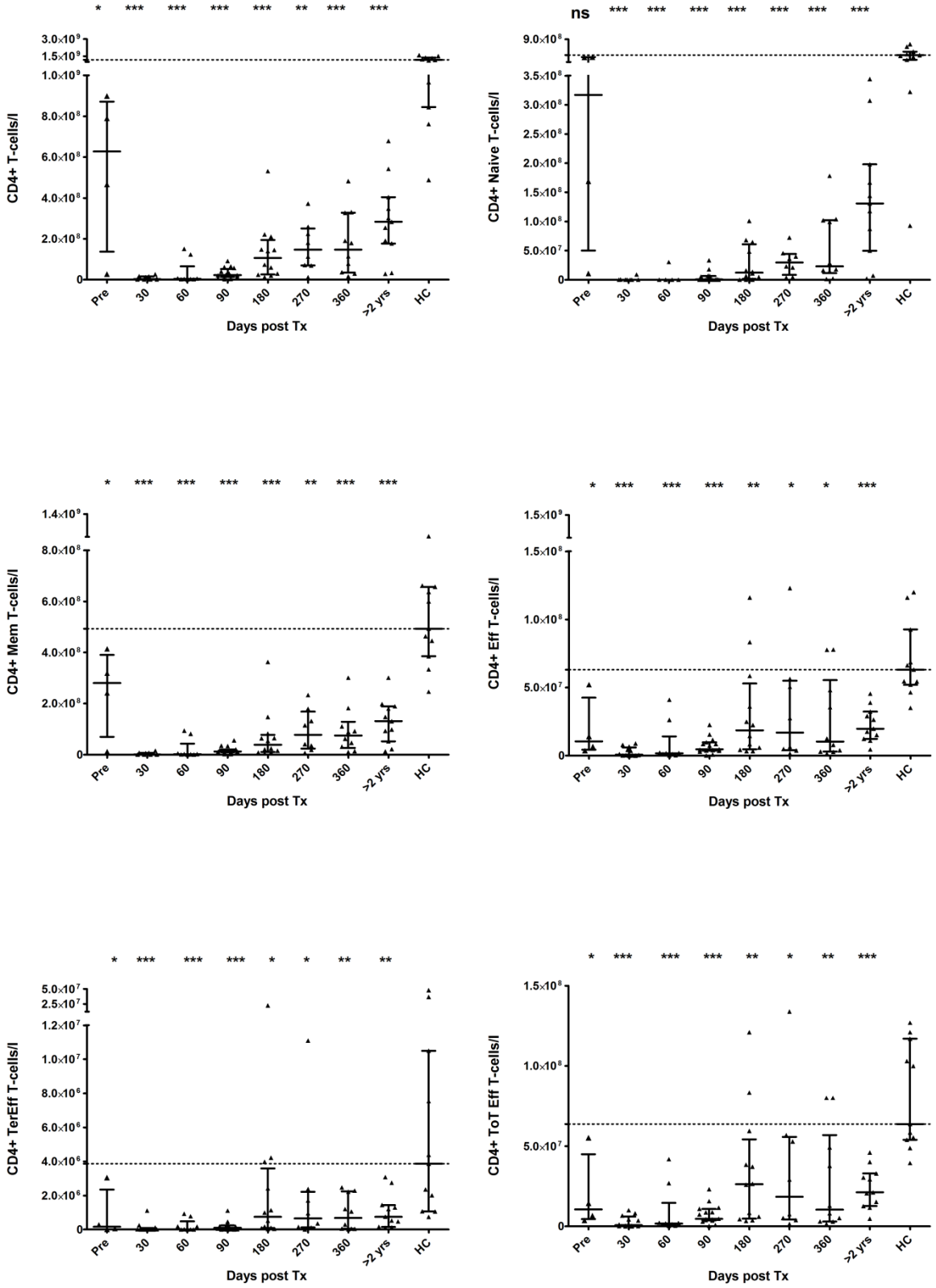

Fig. 15. Total CD4+ lymphocytes (n° of cell/mmc), and relative subsets (Naïve, Memory and Effector cells) after FCC HSCT. Profund CD4+ cytopenia is present, with number remaining below normal one year after transplant. Frequencies compared with healthy control group by Mann-Whitney U-test.
*** = p<0,05 ; ** = p<0,005; *** = p <0,0005; NS = p>0,05**

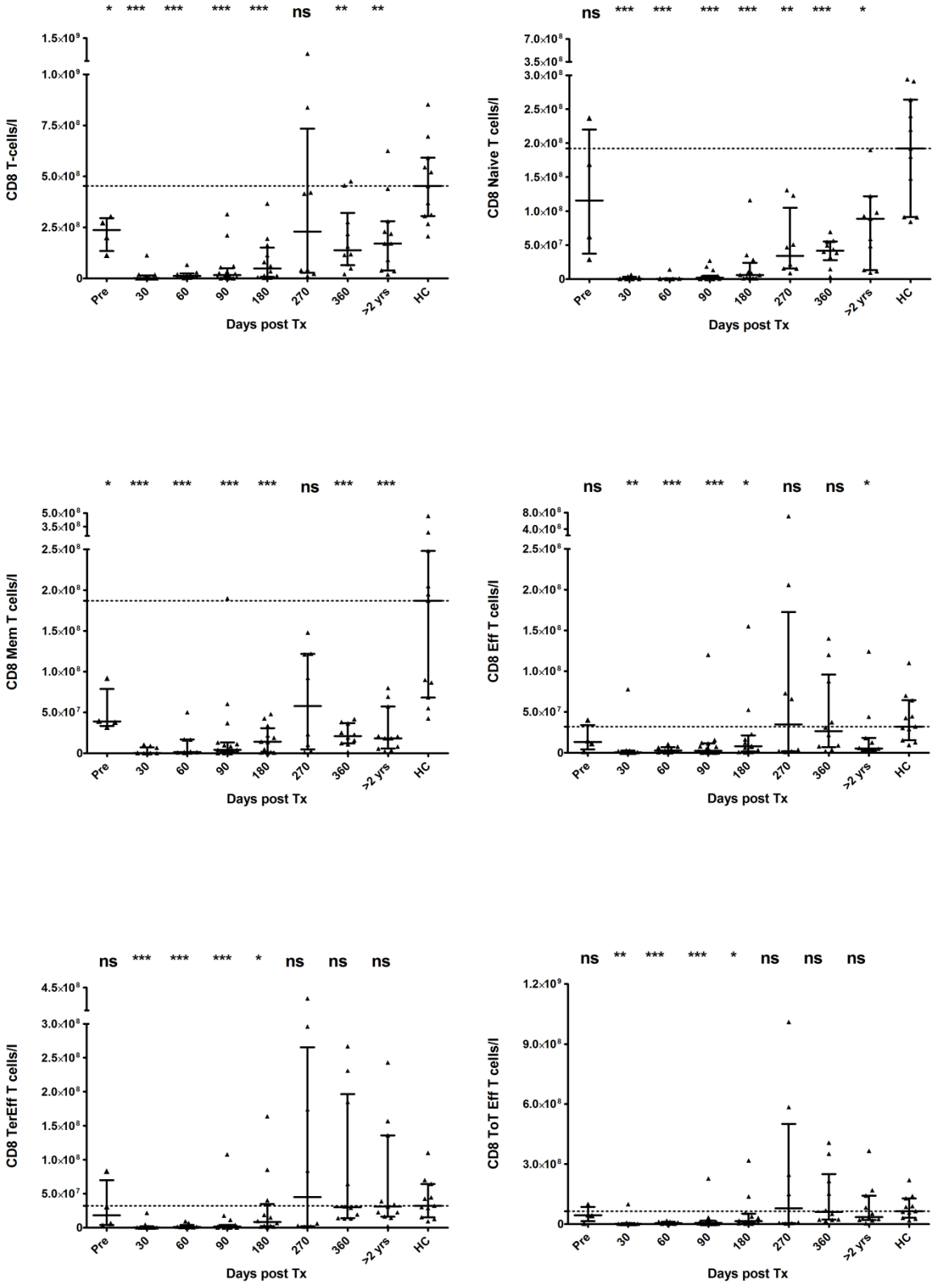

Fig. 16. Total CD8+ lymphocytes (n° of cell/mmc), and relative subsets (Naïve, Memory and Effector cells) after FCC HSCT. Even for CD8+ profund cytopenia is observed, but a relative recover of effector fraction in later timepoints is present. Frequencies compared with healthy control group by Mann-Whitney U-test.
*** = p<0,05 ; ** = p<0,005; *** = p <0,0005; NS = p>0,05**

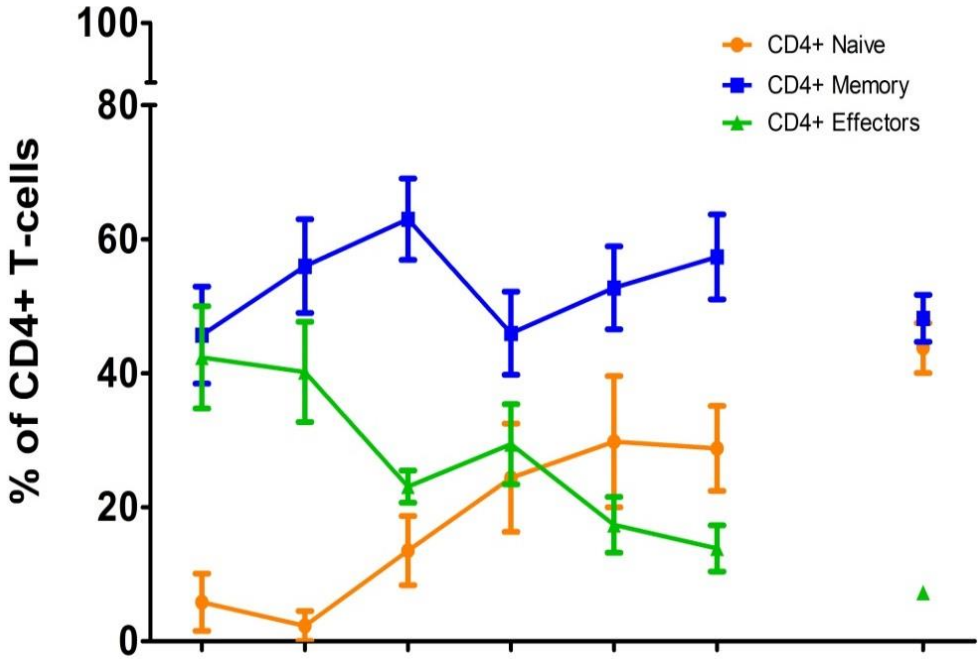

Fig. 17. Relative recovery (% of lymphocytes/mmc) of CD4+ T-cells after FCC. CD4+ T-cells recover to a proportionally normal composition 1-year after HSCT.

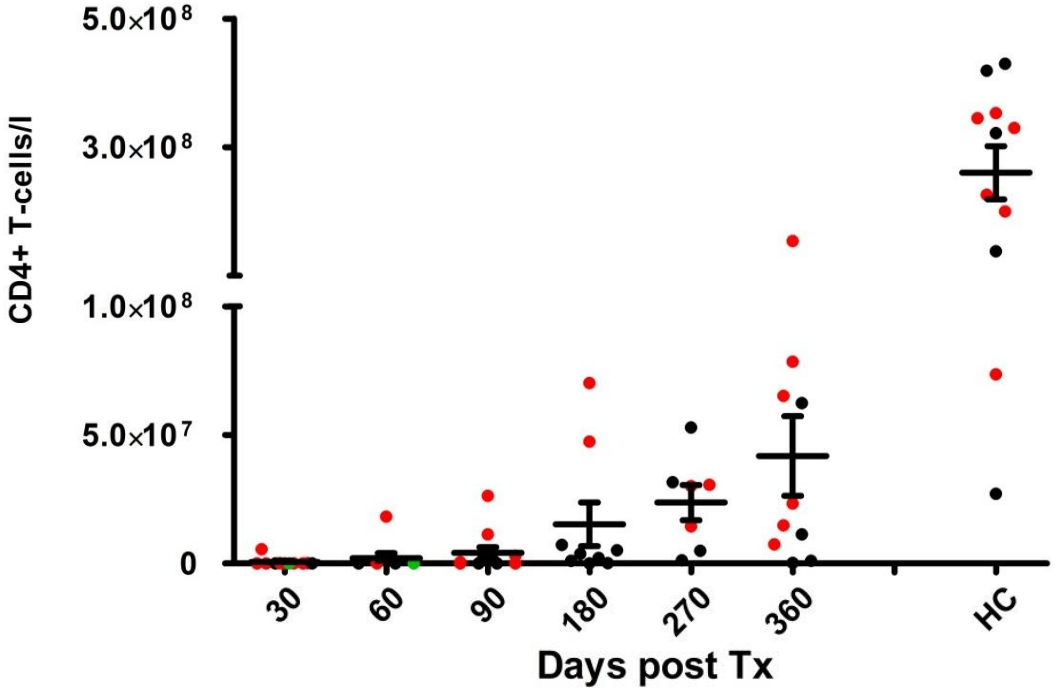

Fig. 18. Recover of CD4+ recently thymic emigrant after FCC highligths presence of central tolerance in AA patients.

Composition of CD8+ T-cell subset population remained skewed toward effectors cells, with a relatively defective number of naïve cells across one year after transplant. As consequence, level of CD8+ Effectors T-cells proportion observed was higher than normal (57,2% of CD8 T cells compared to 9,3% for healthy individuals, p<0,005) highlighting a potential effect of antigenic stimulation on CD8+ T-cell reconstitution (Fig. 19).

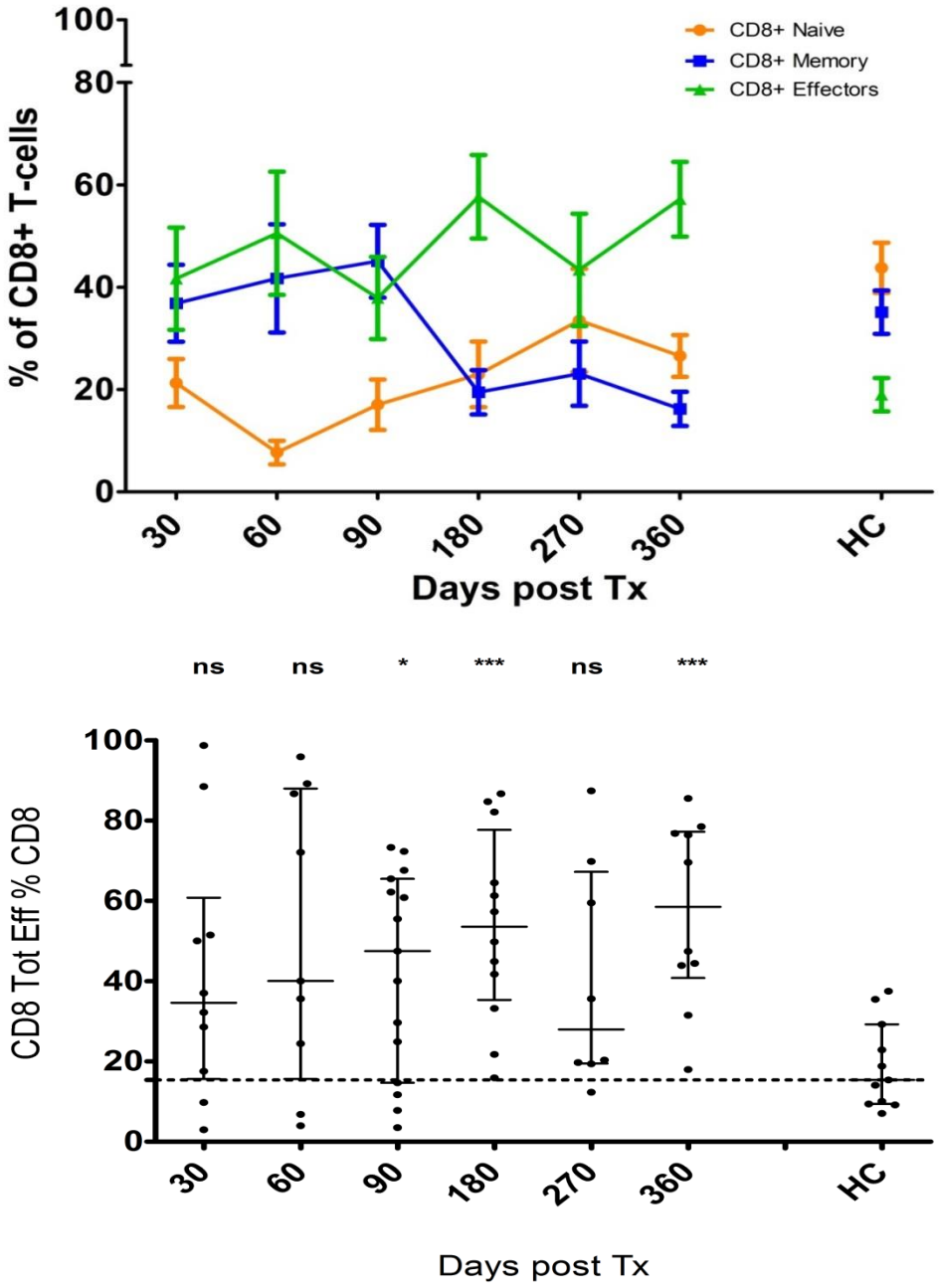

Fig. 19. Relative recovery (% of lymphocytes/mmc) of CD8+ T-cells after FCC (See on top). CD8+ T-cells remain skewed due to augmented proportion CD8+ Effector T-cell, which persists at 1-year after HSCT (See below).
*** = p<0,05 ; ** = p<0,005; *** = p <0,0005; NS = p>0,05**

6.5 Lymphocytes subsets chimerism analysis

Given CD3+ persistent mixed chimerism status observed in this cohort after transplantation, T-cell subsets from five patients were isolated by fluorescence-activated cell sorting, and chimerism status assessed specifically. This analysis revealed that mixed T-cell chimerism at day 360 was principally due to persistence of patient CD8+ T-cells, with notable contribution of the Effector subset. Follow-up analysis of three patients at >2 years after HSCT showed that the mixed chimerism profile within T-cell subsets remained stable across time (Figure 20).

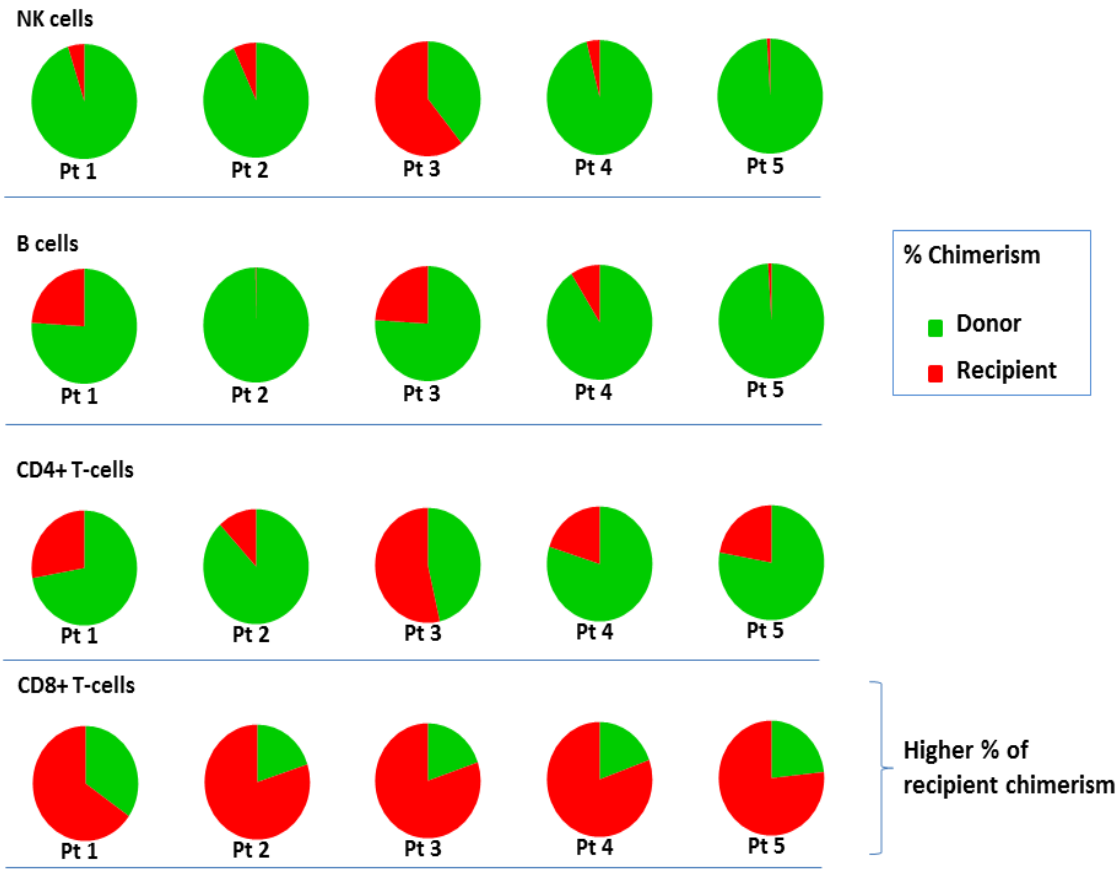

Fig. 20. Lymphocyte subset chimerism status at 1-year after FCC HSCT. Proportion of donor and recipient cell remain significantly different within different subsets.

A significant correlation was observed between lower donor T-cell chimerism at day 360 and CMV reactivation or EBV viremia early after HSCT (p=0.036), suggesting that antigen-driven expansion of virus-specific patient CD8 effector T cells may substantially contribute to persistent mixed T-cell chimerism (Figure 21).

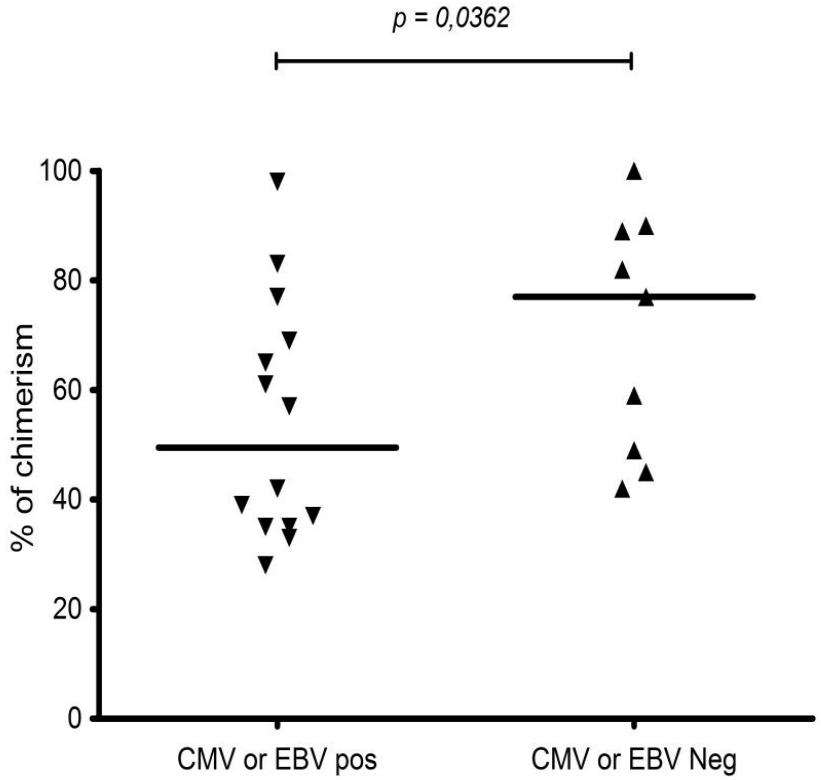

Fig. 21. Dot plot showing chimerism level (with median bars) according to Viral reactivation in FCC transplanted AA patients .

However, analysis of PBMC from one HLA-A*02:01 CMV+ patient with HLA-A*02:01-NLV-pentamer lead to show T-cell specific CMV reactivity did not show high frequencies of CD8 T cells specific for this single epitope (fig. 22).

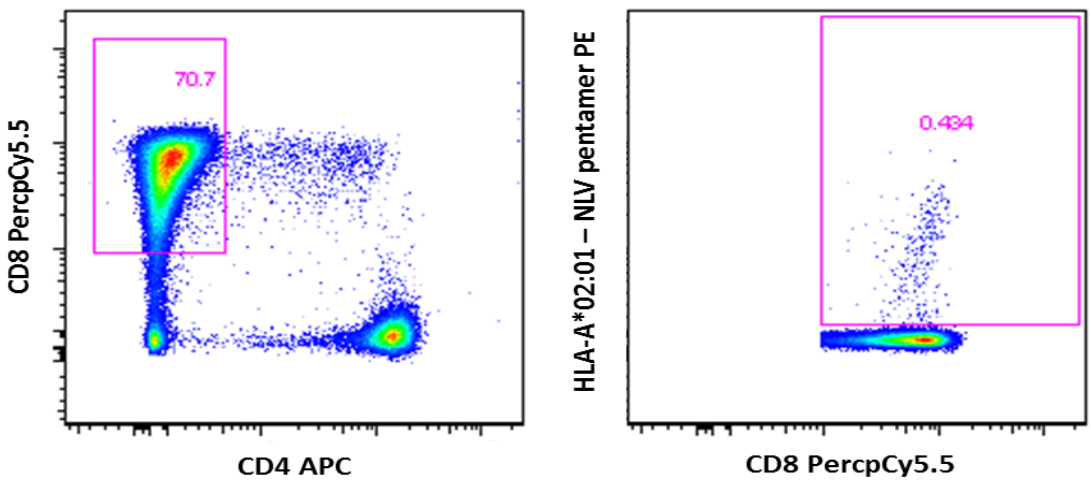

Fig. 22. Experiment showing HLA-A*02:01-NLV-specific CD8+ T-cells .

6.6 Regulatory lymphocytes subsets after HSCT

Sustained co-existence of donor and patient T cells and low rates of GVHD indicates conditions favoring mutual tolerance, therefore we looked for the potential role of cells with immunomodulatory properties after transplantation.

As discussed above, B-cell recovery was robust and faster than NK- and T-cells, achieving normal numbers in peripheral blood by day 100, with a B-cell/T-cell ratio within peripheral blood remaining significantly higher than normal, especially in the early post-transplant period. Moreover study of B-cell subset dynamics revealed early after HSCT a significantly increased proportion of cells with an immature transitional phenotype ($CD24^{+}$ $CD38^{+,}$ $CD27^{-}$ IgM^{high} IgD^{high} 17.1% compared to 2.7% for healthy individuals, p=<0.005) that recently have been deemed to possess regulatory properties (Fig. 23).

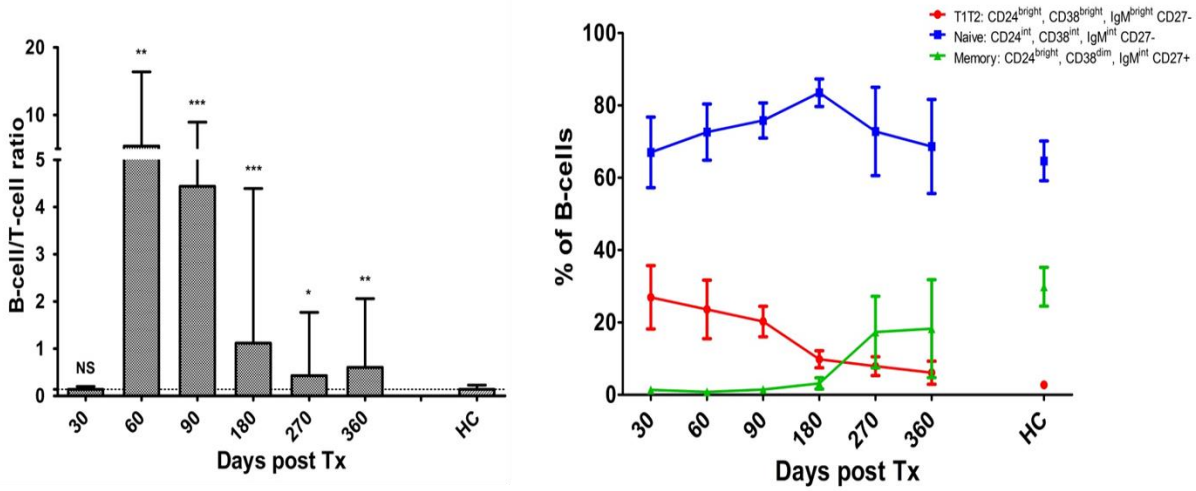

Fig. 23. On left, abnormal B/T-cell ratio after FCC HSCT. On right, dynamics of B-cell subsets.

T1T2 B-lymphocytes have been showed[100] to possess functional suppressive activities through expression of inhibiting cytokines, like IL-10 and TGFβ. Therefore ability to produce IL-10 was assessed in B-cells after in vitro stimulation with CD40L; an higher than normal proportion of IL10-producing B-cells was found in these patients (14.6% compared to 6.3% for healthy individuals, p=0.013), consistent with the known immunosuppressive activity of immature B-cells (Fig. 24).

Furthermore, we looked for presence of regulatory CD4+ T-cells (CD4+ $CD25^{high}$ $CD27^{+}$ $FoxP3^{+}$), which normally suppress the activity of effector T-cells, and consequently, could have a beneficial role in creating a tolerant post-HSCT environment and in controlling GvHD. Enumeration of CD4+ CD25high Foxp3+ T cells showed that the numbers of these regulatory cells after transplantation remained lower than normal across all the post-transplant period, as expected by low T-cell number within these patients. However proportions of regulatory T-cells within the CD4+ T-cell population remained normal (range 4.2-7.4% compared to 5.3% in healthy controls), suggesting a potential balanced effect of these cells towards effector T-cells compartment (Fig. 25).

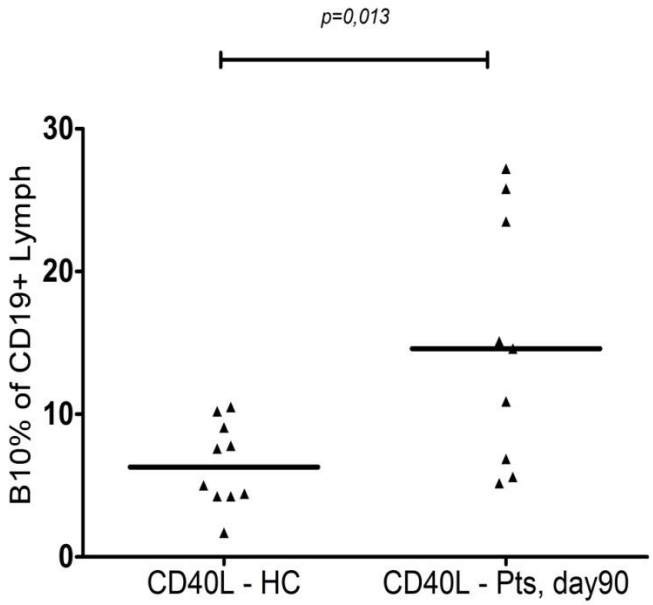

Fig. 24. IL10+ B-cells after FCC HSCT. B10+ cells are presented as percentage of B-cells. Level are compared with healthy controls (HC).

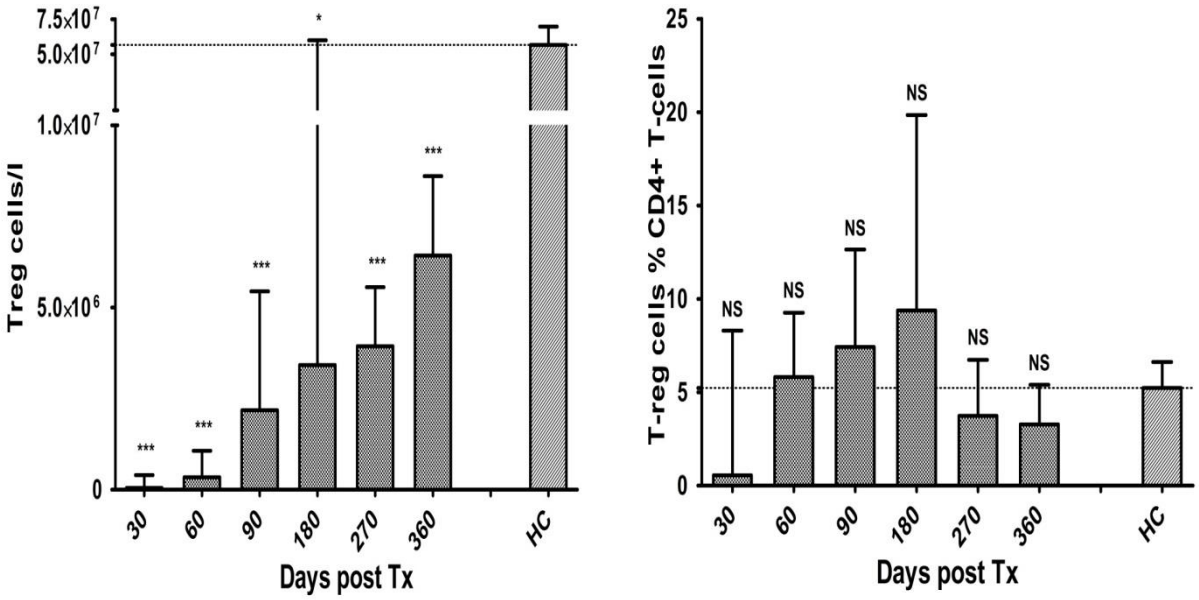

Fig. 25. T-regs reconstitution after-HSCT. On right, T-regs frequencies. On left, T-regs proportions.

7. DISCUSSION

At current state, HSCT is only long-term curative option for patients affected by AA. Aims of allogeneic transplantation in this setting should be different from aims for treatment of other hematological conditions, like Acute Leukemia. As a matter of fact, AA remains an non-neoplastic disease for which stem cell transplantation should be used not only for gaining a complete hematological response, but actually for achieving a complete cure process trough preservation of patients long-term quality of life, including focusing on specific issues like reduced late toxicities (i.e. increased risk of cardiovascular disease or second cancers) and preservation of fertility in younger people. In this perspective, reduction of complication classically associated with HSCT in AA, like augmented incidence of graft rejection, and GvHD, especially chronic, remain an extremely compelling object of research.

Addition of a T-cell lympho-depleting agent to conditioning in order to prevent GvHD, and adoption of reduce intensity conditioning (RIC) chemotherapy regimen for older patient, in order to reduce transplant-related toxicities trough a reduced dose of chemotherapy delivered, have been unanimously recognized as successful achievement in this direction.

Several evidences suggested that FCC conditioning regimen, combining the extremely lympho-depleting activity of monoclonal anti-CD52 antibody Alemtuzumab with a RIC chemotherapy including FLU and Cy, is able to satisfy all the above conditions and guarantee low rates of long-term post-transplant side effects (In competition with other conditioning regimen aforementioned in the introduction like Cy200-ATG and FCA). Major concerns about wide spreading of FCC in transplant practice for AA persist due to relatively short follow-up information available in the experience reported in literature, and absence of immunological data supporting validity of this regimen from a biological point of view. Our study provides good answers to both these two kind of extremely important questions.

First of all, a consistent and homogeneously treated number of patients (N = 45) is available in this study for survival analysis with a median follow-up time superior to 32 months, which is quite exceptional considering rarity of the disease. Excellent survival rate are confirmed (See fig. 8,9,10), absolutely competitive to the average reported in medical literature, and a reduced incidence of both early and late complication is observed, especially graft failure, which show an incredibly low rate in this cohort (2.2%; see table 5), and chronic cGvHD, which appear limited in terms of number of patient affected, grade severity and of organ damaged (Only one patient with severe cGvHD and exclusive skin involvement; see table 5). These features are even more interesting if we consider that PBSC is the most commonly used source of HSC, and MUD is most frequently used type of donor. As reported in the introduction, both these factor should be associated with higher incidence of GvHD and graft failure, with a direct reflection on survival. However all these problems seem apparently resolved in our FCC cohort. More interestingly, a consistent part of patients remain persistently mixed chimeric, even more than one year after transplant, when cyclosporine is completly suspended (Fig. 7). These feature is extremely interesting if we considered that a mixed chimerims status in AA is a consistent risk factor for graft rejection, in particular when a dropping tendency is observed. Nevertheless, this tendency is not observed in our patients, where a pacific co-existence between donor and recipient cells seems to be established, which actually suggest achievement of a tolerant immune environment. These data are concordant with the fact that a significant risk for cGVHD in our cohort is the rapid

establishment of full donor chimerism status at day +90, suggesting a not well counterbalanced immune activity of donor cells in these patients.

Our immunological data give some intel about all these peculiar clinical aspects occurring after FCC transplant. Firstly, lympho-depleting activity of this Alemtuzumab-based regimen is so strong that lymphocyte counts do not show complete recover even one year after HSCT. Particularly, this condition affects the T-cell compartment, which a huge amount of data available in literature indicate as responsible for triggering and perpetuating post-HSCT immune phenomena as graft rejection or GvHD. However in our patients T-cell counts tend to recover across time, with phenomena indicative of potential reducation during recovery.

CD4+ T-cell compartment, for example, shows a continuous improvement in count of Naïve CD4+ T-cells across time. Most of these cell express markers of recently thymic emigration in younger patients, indicating that thymus is active after transplant, and probably has a role in reeducating lymphocyte not to trigger immune reaction against host antigens, how actually GvHD is. On the contrary, CD8+ T-cell compartment do not show generation of big counts of Naïve CD8+ T-cells, while an augmented proportion of CD8+ Effector cells, which currently constitute the armed branch of the immune system, is predominant. This phenomena is so pronounced that physiological CD4/CD8 ratio is altered. This condition remain quite unusual, if we consider that CD8+ Effectors expansion should be avoidable in AA, given the immune pathogenesis of the disease (see Introduction). Nevertheless, graft failure, which we can actually consider disease relapse, is not present with an augmented rate in our patients (Actually is the opposite). A potential explanation is that only few CD8+ clones with a stereotypical TCR can expand after FCC HSCT, and that these cell are not able to promote or perpetuate GvHD. Viral infection occur at an high rate in our cohort, even if remain clinical irrelevant due to intense clinical monitoring (See table 5). Lower chimerism level in patients with active viral reactivations (see fig. 21), and specifically high level of recipient cell in CD8+ T-cell fraction (see fig. 20), suggest that the higher incidence of viral infection caused by Alemtuzumab are probably able to expand TCR-restricted cells of recipient origin; these cells are not able to attack the HSC due to their viral specificity, nor to promulgate GvHD due to their autologous nature. Finally presence of regulatory element (T-regs, and IL10+ B-regs) may promote the forced coexistence between donor and recipient cell (persistent mixed chimerism status, eventually started by viral stimulation), and may facilitate the persistence of a suppressive/permissive post-HSCT immune environment. Of note, even if this hypothesis was not confirmed when we looked for NLV-CMV-peptide-specific CD8+ T-cells, it was a single experiment, while is probable that in these patients different epitopes, from different peptides, and probably even form different viruses, need to be explored, before drawing any conclusion, given the elevate number of viral infections observed in clinic (see table 5).

Finally our study confirm excellent clinical outcome for patients receiving HSCT for AA with FCC regimen, and for first time provides critical hints on efficacy on this treatment approaching the dynamics of immune reconstitution after transplantation. Advancement of knowledge in this filed would eventually provide new understandings in clinical management of these patients, gaining higher rates of successful cure for this rare and difficult disease.

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10. PUBLICATIONS IN EXTENSO

PhD Period

1. **Grimaldi F**, Ghandi S, Risitano AM. *Epidemiology of acquired bone marrow failure syndrome – Book chapter.* On the behalf of SAAWP-EBMT, 2016. **Publication in press.**

2. Risitano AM, Marotta S, Calzone R, **Grimaldi F,** Zatterale A; all RIAF Contributors. *Twenty years of the Italian Fanconi Anemia Registry: where we stand and what remains to be learned.* Haematologica. 2016 Mar;101(3):319-2.

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Intership period (Pre-PhD)

1. Picardi M, Zeppa P, Ciancia G, Pettinato G, **Grimaldi F,** Fabbricini R, Mainolfi C, Pane F. *Efficacy and safety of rituximab treatment in patients with progressive transformation of germinal centers after Hodgkin lymphoma in complete remission post-induction chemotherapy and radiotherapy.* Leuk Lymphoma. 2011 Nov;52(11):2082-9.

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ARTICLE

Twenty years of the Italian Fanconi Anemia Registry: where we stand and what remains to be learned

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ABSTRACT

The natural history of Fanconi anemia remains hard to establish because of its rarity and its heterogeneous clinical presentation; since 1994, the Italian Fanconi Anemia Registry has collected clinical, epidemiological and genetic data of Italian Fanconi Anemia patients. This registry includes 180 patients with a confirmed diagnosis of Fanconi anemia who have either been enrolled prospectively, at diagnosis, or later on. After enrollment, follow-up data were periodically collected to assess the clinical course, possible complications and long-term survival; the median follow up was 15.6 years. The main goal of the study was to describe the natural history of Fanconi anemia, focusing on the following variables: family history, disease presentation, development of hematological manifestations, development of malignancies, occurrence of hematopoietic stem cell transplantation and survival. Typical morphological and/or hematological abnormalities and/or growth retardation were the most common manifestations at diagnosis; the majority of patients (77%) exhibited hematological abnormalities at the initial presentation, and almost all (96%) eventually developed hematological manifestations. More than half of the patients (57%) underwent a bone-marrow transplant. The occurrence of cancer was quite rare at diagnosis, whereas the cumulative incidence of malignancies at 10, 20 and 30 years was 5%, 8% and 22%, respectively, for hematological cancers and 1%, 15% and 32%, respectively, for solid tumors. Overall survival at 10, 20 and 30 years were 88%, 56% and 37%, respectively; the main causes of death were cancer, complications of the hematological presentation and complications of transplantation. These data clearly confirm the detrimental outcome of Fanconi anemia, with no major improvement in the past decades.

Introduction

Fanconi anemia (FA)[1] is a rare inherited hematological disorder biologically characterized by hypersensitivity to DNA cross-linking agents. FA is mainly an autosomal recessive disease (except the rare X-linked FANC-B form), which was first reported in 1927 by the Swiss pediatrician Guido Fanconi as familial, infantile anemia.[2] FA is now defined as a chromosomal instability (CI) syndrome, and it shows a wide clinical and genetic heterogeneity. Indeed, genetically FA can be caused by mutations in at least 18 different genes, mostly cooperating in a pathway which has not yet been fully elucidated. These gene products somehow interact with proteins encoded by genes which, when mutated, cause other CI syndromes, such as Ataxia Teleangiectasia, Bloom syndrome, and Nijmegen Breakage Syndrome.[3] All these proteins cooperate in a pathway which appears to be involved in DNA and oxidative stress damage repair.[4] The FA cellular phenotype is characterized by a G2 cell cycle phase delay[5] and by CI, typically appearing as characteristic rearrangement

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figures (triradial and quadriradial figures made by non-homologous chromosomes). The CI is both spontaneous[6] and/or induced by alkylating DNA cross-linking agents, such as mytomicin C (MMC)[7] or the more specific diepoxybutane (DEB);[8] the cytogenetic DEB-test makes the FA diagnosis possible in those patients not showing typical malformations or still asymptomatic, and also in patients not showing spontaneous CI at standard cytogenetic evaluation.

The phenotype of FA patients is largely heterogeneous, since the natural history of the disease entails different clinical manifestations which may either be present at birth, or develop later during the course of the disease. Clinically, FA patients present bone marrow failure at various times in life, typically beginning in childhood as platelet deficiency, and then progressing to pancytopenia,[1] eventually leading to life-threatening complications. FA patients can show variable congenital malformations[9] and are prone to hematologic and solid neoplasias, which are ultimately the leading cause of death.[1,4,10-12] At present, hematopoietic stem cell transplantation (HSCT) represents the only effective treatment for FA,[13] although unfortunately it cannot improve the patient's growth rate or reduce the propensity to develop non-hematological cancers.

FA is a rare disease, with an incidence rate of 0.1-0.5 new cases for every 100000 newborn children;[1,14] thus, large multicenter studies are needed to better describe the natural history of the disease. Ideally, to prevent any bias, such studies should include all patients diagnosed with FA in a broad but well-defined geographic area, possibly with a prospective collection of data and an adequate long-term follow-up. Until now, the most reliable data on FA has come from the International Fanconi Anemia Registry (IFAR)[15,16] and some national Registries, mostly the North American Survey of Fanconi Anemia (NAS)[12,17] and the German Fanconi Anemia Registry (GEFA).[18] In a rare and highly heterogeneous disease such as FA, it is very difficult to establish the natural history of the disease, and even more difficult to organize research projects which require the collection of samples from patients showing common features. Moreover, some significant differences are possible among different populations, due to the existence of geographic isolates or genetic derives. A specific national registry, collecting patients' clinical, epidemiological, genetic and familial data, becomes a powerful tool, which creates for physicians and the scientific community the possibility of better knowing the disease, hence preventing misdiagnosis and delayed diagnosis. A national registry also creates a network that facilitates the participation of patients in research projects and clinical trials.

To fulfill the need for a National Database including most (if not all) FA patients diagnosed in Italy, in 1994 some of us started "*Il Registro Italiano Anemia di Fanconi*" (RIAF), which translates as 'The Italian Fanconi Anemia Registry'.[19] This project was established within the Italian Public National Health System (NHS), at the Genetic Unit of the local health unit "ASL Napoli 1", taking advantage of their established expertise in diagnostics and genetic counseling for CI syndromes. The aims of the project were: i. to create a National database, recording all Italian FA cases; ii. to collect information about the epidemiology of FA in Italy, as well as about its natural history and therapeutic interventions; iii. to promote a robust scientific network among health workers (physicians, but also scientists) dealing with FA in Italy, eventually increasing awareness about the disease and promoting the strongest possible collaboration among Italian physicians and scientists; iv. to provide patients and their families with an established national network for the diagnosis, treatment and follow-up of FA patients; v. to share with the Italian authorities all the information above, aiming to assess the real impact of FA on the Italian NHS, eventually promoting further health policy strategies. Herein we report on the 20-year experience of this Registry, focusing on the natural history of the disease, frequent therapeutic modalities and long-term outcomes of FA patients. The major aim of this work was to identify possible factors affecting the survival of FA patients, as well as to identify possible changes in outcome emerging over the two decades of this study.

Methods

Study design and data handling

The RIAF was officially established in 1994 as a prospective, non-interventional study, approved by the local Government (serving as the institutional review board, IRB) and operated according to National laws within the NHS. The program was approved and funded by the Regional Government (Regione Campania), and supported by the Italian Association for Research on Fanconi Anemia (AIRFA). All patient data were collected through dedicated case report forms (CRFs), designed by the geneticists working at the ASL Napoli 1, and further developed thanks to the contributions of collaborating physicians (listed in the Appendix). In accordance with the Declaration of Helsinki, before enrollment all patients or their parents/guardians gave written, informed consent, after discussing the RIAF aims and policy and their own rights with an authorized delegate (geneticist or physician), as listed in a written information sheet. Follow-up CRFs were periodically filed by the geneticists or the physicians, through a continuous sharing of critical information with both treating physicians and patients or their families. The CRFs and informed consent were approved by the local Government/IRB. The data were stored both on paper and in digital records, strictly protected, accessible only to the authorized staff and always made anonymous for publications or sharing with other researchers.

Inclusion criteria, enrollment and data collection

Patients were enrolled only after a positive DEB or MMC test, for the most part carried out or confirmed in our laboratory. The chromosome breakage assay was always performed on peripheral blood cells. Patients were enrolled either at the time of initial diagnosis, or later, during the course of the disease, according to patients' and/or parents' decisions. Following informed consent, family history and medical information were recorded by the treating physicians, according to the specific CRFs. Given the epidemiologic purpose of the RIAF, the data of patients belonging to non-Italian ethnic populations were collected separately and are not considered here. Indeed, the patients geographic designation was established on the basis of their parents' and grandparents' birthplaces (Caucasian ethnicity and proven Italian descent covering at least two generations); the same criteria were used to assign a patient to a specific Italian Region.

Data analysis and statistics

Statistical analysis was performed on the population of 180 patients (fetal losses and miscarriages were excluded), focusing on the following categories: family history, disease presentation, hematologic manifestations, HSCT, treatment impact on survival,

malignancies, overall and cancer-free survival, and causes of death. Standard descriptive statistical tests were applied as appropriate, using SPSS software (PSP, Bologna, Italy). Student's t-test, Mann-Whitney test and Fisher's exact test were used for most descriptive analysis. The time to developing specific disease presentation (i.e., hematological presentation, hematological malignancies, solid tumors) was presented as cumulative incidence, using a competing risk approach, with birth treated as the FA onset date; death and HSCT (the latter only for hematological presentation and hematological malignancies) were considered as competing risks. The Kaplan-Meier curve was used to estimate overall survival; again birth was considered as the FA onset date. The following variables were tested for a possible impact on survival: gender, date of birth, age at diagnosis, all congenital abnormalities (presence, total number, type; with and without skin abnormalities), hematological presentation (at diagnosis, or at any time), hematological malignancies, solid tumors (all together and head/neck) and HSCT. Univariate and multivariate analyses were performed using a Cox regression model on all patients, as well as separately on transplanted and non-transplanted patients.

Results

Diagnosis and genetics of FA

A preliminary diagnosis of FA was made by treating physicians, based on clinical presentation at birth, or later on; in some patients their family history was the main reason to hypothesize the presence of FA. The diagnosis of FA was based on a standard chromosome breakage test by exposure to DEB or MMC, performed on peripheral blood samples. Given the possible challenges in the diagnosis of CI, all tests were confirmed at the Genetic Unit of the ASL Napoli 1, or other laboratories with specific expertise for the diagnosis of FA, eventually limiting subjective interpretations and inter-laboratory technical variability. Lymphoblastoid cell lines were established for research aims and as diagnostic positive controls. A single DEB test was sufficient for the diagnosis in the majority of cases; however, the protocol adopted at the Genetic Unit of the ASL Napoli 1 was used to confirm the diagnosis on two different samples, allowing a robust consistency of data. Between 1989 and 2014, out of a total of 1340 DEB tests performed on 1185 subjects, the number of positive tests was 206 (for 135 patients). Notably, a prior misdiagnosis was proven in 11 patients: in 7 cases a previous diagnosis of FA was not confirmed, whereas in 4 patients the diagnosis of FA was missing. Mosaicism was suspected in 9% of patients.[20] They showed chromosome breaks in <40% of their cells, but typical DEB-induced rearrangements were demonstrated; CI testing performed on different tissues, together with clinical, family and/or molecular data, confirmed the FA diagnosis. Patients lacking a confirmatory positive chromosome breakage test were not enrolled in the RIAF, irrespective of their clinical presentation; thus, possible revertant phenotypes may be underrepresented in this cohort. Complementation groups were available for 55 patients; the most common complementation group was A (91%), followed by G (5%) and D2 (4%) (*Online Supplementary Table S1*).

Subject characteristics

Between 1994 and 2014, a total of 180 patients were included in the RIAF, belonging to 151 distinct families (median number of affected subjects per family was 1, range 1-4; Table 1); a few (n=3) cases of miscarriage diagnosed as FA (by DEB and/or molecular tests on amniocytes or chorionic villi) were also recorded, but were not included in this study. The geographical distribution was spread throughout the country, even if a significant number of patients were from the North-East or the South of Italy;[21] however, we were unable to identify any founder effect. The characteristics of enrolled patients are described in Table 2. There were 94 (52%) male and 86 (48%) female patients, with no statistical difference in gender. The median age at diagnosis was 7.48 years; when patients were divided according to the date of birth, by quartiles (≤1980, 1981-1987, 1988-1995, ≥1996), the age at diagnosis was significantly lower in patients born in more recent periods (Mann-Whitney test, $P<0.001$; Figure 1A).

Family history

Family history was carefully collected for all patients included in the registry; parents consanguinity was recorded in 20 of the 151 families (14%; Table 1). For each family (considered as two parents), the median number of affected children was 1 (range 1-4), the median of unaffected siblings (all confirmed by DEB test: all alive siblings were tested even as potential HSCT donors) was 1 (range 0-10); the median of miscarriages was 0 (range 0-3). Globally, in the 151 families enrolled we recorded 310 babies, of whom 183 (60%) were FA cases (180 enrolled in the RIAF and 3 miscarriages), and 127 were unaffected siblings. Thirty-seven of the patients included in the registry reported a family history of FA. We also looked for the occurrence of hematological disorders and malignancies in the relatives of enrolled FA patients (up to the second degree of kinship); family history for cancer (taking into account up to the second degree) was 56% (85 out of 151). Family history for hematological disorders was demonstrated in 19% of patients. Morphological abnormalities in some relatives were recorded in 19% of patients.

Disease presentation

In the majority of patients the diagnosis was suspected based on typical morphological and/or hematological abnormalities and/or growth retardation. As detailed in

Table 1. Characteristics of the 151 families.*

Variable		151 Families (%)
Consanguinity between parents	None	131 (86)
	1st degree	13 (9)
	2nd degree	3 (2)
	>2nd degree	4 (3)
N. of affected children°	1	124 (82)
	2	22 (14)
	3	4 (3)
	4	1 (1)
N. of not affected children	0	35 (23)
	1	65 (43)
	2	36 (24)
	3 or >3	15 (10)
N. of miscarriages	0	122 (81)
	1	21 (14)
	2	7 (5)
	3 or >3	1 (<1)

**For this analysis, a family is intended as two parents with their children; data are expressed by family; °including abortions with confirmed FA.*

Table 2, congenital abnormalities were demonstrated in 90% of patients at the time of diagnosis; the most common were the typical abnormalities of skin pigmentation, which affected 96% of RIAF patients (Table 2); skeletal abnormalities were also very frequent (57%). Other common congenital abnormalities involved growth retardation (39%), the central nervous system (35%), the urinary system (34%), the genital tract (18%), the gastrointestinal tract (13%), the eyes (12%), the endocrine system (9%) or the cardiovascular system (7%). Hematological manifestations were defined according to the definition of aplastic anemia[22] and in accordance with the WHO 2008 classification of myeloid malignancies.[23] The majority of patients (77%) exhibited some hematological abnormalities at diagnosis, which in most cases was a mild-to-moderate cytopenia eventually associated with some degree of bone marrow failure (BMF), whereas hematological malignancies (e.g., myelodysplastic syndromes, MDS) and solid tumors were very rarely observed at diagnosis (Table 2). Thanks to the long-term follow-up of the enrolled patients, we were able to assess the further course of the disease with the development of the most common complications of FA, as well as the impact of different factors on survival.

Time to hematological manifestations: bone marrow failure and hematological malignancies

Even if hematological abnormalities were present at diagnosis in only 77% of cases, a total of 172 (96%) of FA patients enrolled in the RIAF had some hematological manifestations during their disease course; in almost all cases (172, 96%) this included cytopenia due to BMF, whereas a hematological malignancy (mostly MDS or acute leukemia, n=9 and n=4, respectively) was recorded in 8% of patients (see Table 3). As expected, in many cases an initial BMF progressed to either MDS or more aggressive hematological cancers; 1 MDS, 1 leukemia and 1 lymphoma patient did not evolve from a previous BMF. Considering death and HSCT as competing events, the cumulative incidence of any hematological disorder was 62%, 88% and 94% at 10, 20 and 30 years respectively, whereas the incidence of hematological malignancies was 5%, 8% and 22% at 10, 20 and 30 years, respectively. 'The cumulative incidence of the first hematological presentation and of the first hematological malignancy is depicted in Figure 1B.

Time to hematopoietic stem cell transplantation

The development of a hematological presentation is the main indication for HSCT in FA patients; indeed, more than half of the patients enrolled in the RIAF (102 out of 180, 57%) had received a HSCT from either a non-affected sibling or matched unrelated donor (Table 3). The first HSCT was performed from a non-affected sibling in 38% of cases, from a matched unrelated donor in 48%, and quite rarely from cord blood (4%) or a mismatched related donor (0.9%). The cumulative incidence of HSCT in our patient cohort was 33%, 64% and 72% at 10, 20 and 30 years, respectively, as depicted in Figure 1B. The age at transplant was significantly different according to the date of birth cohorts, since patients born in more recent years were transplanted earlier (Mann-Whitney test, $P<0.001$; Figure 1C). Since National and European Registries collecting transplant-specific information exist,[15,16] in the RIAF we decided not to duplicate this information. A formal analysis of HSCT outcome in these patients is beyond the scope of this study. However, follow-up data on survival and the possible development of malignancies were also collected for those RIAF patients who received a HSCT.

Cumulative incidence of solid tumors

A total of 27 solid cancers were diagnosed in 20 of the 180 RIAF patients (11%); a few patients experienced multiple cancers. The most common sites of cancer were the head and neck (n=12, 44% of all solid tumors), liver (n=3, 11%), breast, thyroid and genital tract (n=2 for each, 7%) (see Table 3 for details). The cumulative incidence of solid tumors was 1%, 15% and 32% at 10, 20 and 30 years respectively, as depicted in Figure 1B. The incidence of all solid cancers and of head and neck tumors was not statistically different between patients who had received a HSCT and those who had not ($P=0.43$ and $P=0.50$, respec-

Table 2. Patient characteristics at diagnosis.

Variable	180 Patients (%)
Gender	
Female	86 (48)
Male	94 (52)
Age (median years at diagnosis)	7,48 (0-37,7)
Family history	
no	143 (79)
yes	37 (21)
Family history for malformations	
no	145 (81)
yes	35 (19)
Family history for hematological diseases	
no	145 (81)
yes	35 (19)
Family history for cancer	
no	79 (44)
yes	101 (56)
Congenital abnormalities	
none/unknown	17 (10)
skin only	16 (9)
*structural abnormalities**	147 (81)
Type of malformation	
skin hyper- hypopigmentation	163 (96)
skeletal abnormalities	103 (57)
growth retardation	70 (39)
central nervous system	63 (35)
renal and urinary tract	61 (34)
genital tract	33 (18)
gastrointestinal tract	23 (13)
eyes	21 (12)
endocrine system	16 (9)
cardiovascular system	12 (7)
Hematological disease	
BMF*	
no	42 (23)
yes	138 (77)
Hematological malignancies^	
no	179 (99)
yes	1 (1)
Solid tumors	
no	177 (98)
yes	3 (2)

**As defined according to the International Agranulocytosis and Aplastic Anemia Study Group; ^As defined according to the WHO 2008 criteria.*

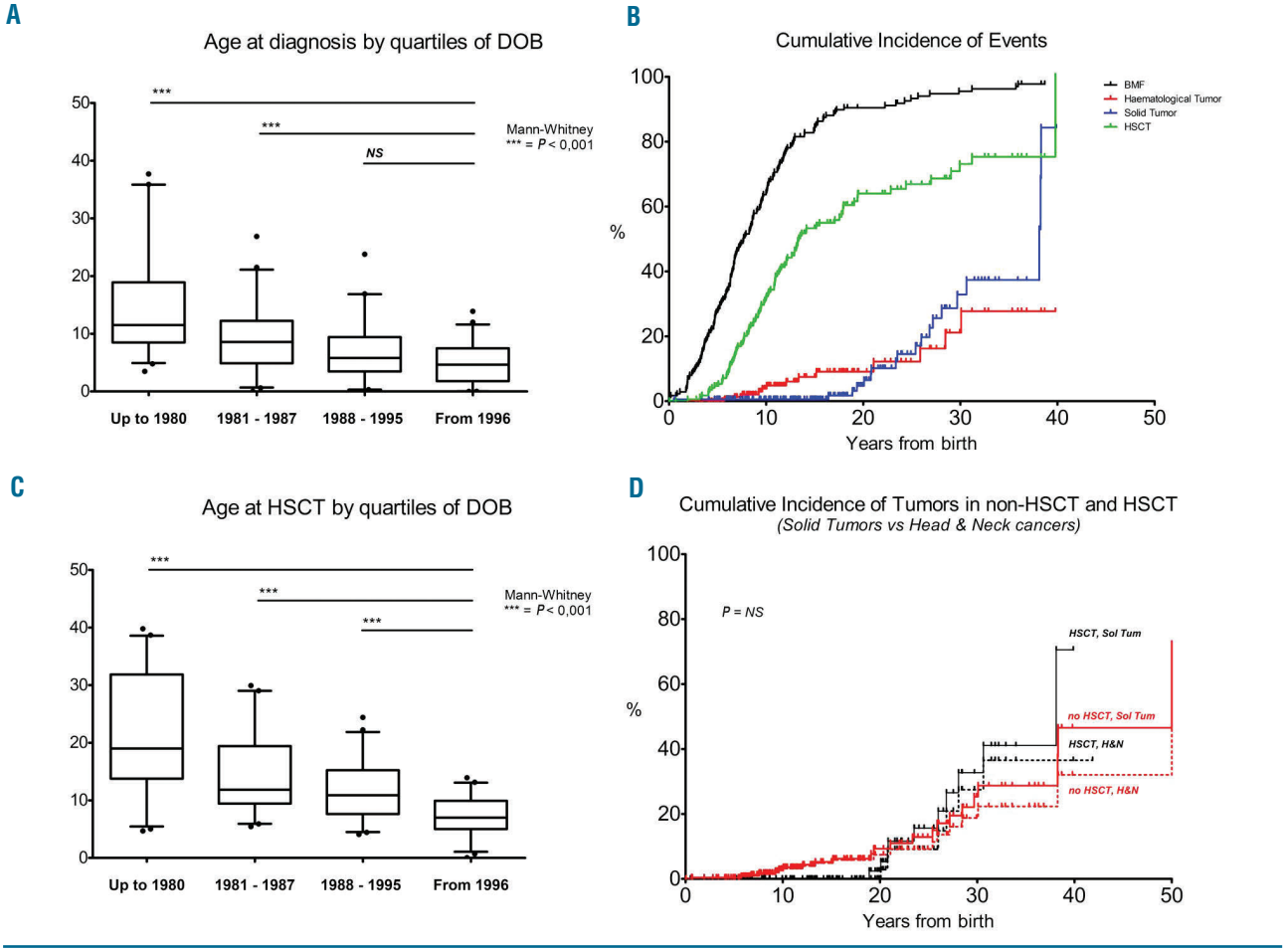
tively; Figure 1D), even if the analysis is limited by the small number of events. In transplanted patients, all but one tumor occurred after HSCT.

Overall survival and prognostic factors

Ninety-four of the 180 patients were still alive at the time of the last follow-up. For all patients enrolled in the RIAF, overall survival was calculated starting from the day of birth. With a median follow up of 15.6 years, median survival was 22.5 years (Figure 2A); probabilities of survival at 10, 20 and 30 years were 88%, 56% and 37%, respectively (without censoring HSCT patients). Looking for the natural history of the disease, when patients who had received an allogeneic HSCT were censored at the time of transplant, the probabilities of survival at 10, 20 and 30 years were 85%, 39% and 24%, respectively (Figure 2B). In univariate analysis, no patients feature affected overall survival, except age at diagnosis and the number of structural abnormalities (excluding skin anomalies; *Online Supplementary Table S2*). In multivariate analysis, an older age at diagnosis (HR=0.873, $P<0.001$) and the presence of more than 10 structural abnormalities (excluding skin; HR=6.504, $P=0.017$) were associated with a better or worse survival rate, respectively. This distinction resulted in statistically significant differences in overall survival based on age at diagnosis (Figure 2C; $P<0.001$) and on higher numbers of structural abnormalities (Figure 2D; $P<0.005$). Notably, BMF at initial presentation, date of birth cohorts and HSCT seemed not to affect survival (Table 4). Indeed, looking at overall survival by quartiles of date of birth there was no improvement in survival over time (Figure 2E; P=n.s.). Similarly, grouping patients by HSCT, the 10, 20 and 30 year survival rate of non-transplanted patients (n=78, median follow-up 15.8 years) were 84%, 49% and 34%, respectively, while those of transplanted patients (n=102, median follow-up 16 years) were 90%, 62% and 40%, respectively (Figure 2F; $P=0.17$). Multivariate analysis was also performed separately on non-HSCT and HSCT patients; in this context, age at diagnosis remained associated with a better survival rate in both groups (*Online Supplementary Table S3*).

Cause of death

Eighty-six of the 180 FA patients enrolled in the RIAF died during their follow-up; the main causes of death are listed in Table 5. As expected, the causes of death were different in patients who had not received an HSCT as compared with those of transplanted patients ($P<0.001$; Chi-square test). Indeed, in non-HSCT patients the main causes of death were related to the underlying disease (i.e., for the most part the hematological abnormalities), such as



1. Time to most common FA complications. (A) Age at diagnosis, according to quartiles of date of birth (DOB); (B) Cumulative incidence of bone marrow failure (BMF), hematological malignancies (MDS and AML; HEM TUM), solid tumors (SOL TUM) and HSCT; (C) Age at HSCT, according to DOB quartiles; (D) Cumulative incidence of solid tumor and of head/neck tumors: HSCT *vs.* no HSCT.

infections (n=14, 33% of total deaths in non-HSCT patients), bleeding (n=8, 18.5%) and solid tumors (n=9, 21%). In contrast, in HSCT patients the majority of deaths were somehow related to treatment complications, such as infections (n=11, 25.5% of total deaths in HSCT patients), graft *versus* host disease (GvHD) (n=11, 25.5%) and other transplant related mortality (TRM) (n=13, 30%). Solid tumors accounted for 9% of deaths (n=4).

Discussion

The RIAF is the first population-based Italian database, run within the Italian NHS, focusing on FA, which is rare in the frequency of the disease, but the most common among inherited bone marrow failure syndromes. Herein we report a comprehensive analysis of all patients included in the registry over the past 20 years with their long-term follow-up, eventually providing a reliable description of the natural history of FA. In our series of 180 prospectively collected patient data, we have shown a median survival of 22 years, which unfortunately has not improved in the past two decades. Our efforts of creating a robust scientific network have increased the awareness of this disease in Italy, eventually leading to objective achievements. Indeed, the diagnosis has come to be made earlier over the past decades, and the time to the only curative treatment – namely HSCT – has decreased. Nevertheless, these improvements in the management of FA patients have not yet resulted in a better survival rate, and even the outcome of patients who have received a HSCT does not appear to be better than that of those who did not. Indeed, in our multivariate analysis, the only factors associated with a better outcome were an older age at diagnosis and a lower number of structural abnormalities, indicating that different clinical phenotypes may have a different life expectancy.

The natural history of FA has been described in previous retrospective studies,[15-18] which have highlighted the heterogeneity of clinical presentation. The RIAF includes only patients with a DEB test confirmed diagnosis of FA, who are unselected for specific disease presentations and have a long-term follow-up. Thus, selection biases (e.g., toward a severe phenotype) should be limited (with the only exception being a possible underestimation of patients beginning with cancers, who might not receive the correct FA diagnosis), eventually leading to a more accurate representation of the natural history of FA. However, like all registry studies, the RIAF suffers from potential limitations, since its completeness and accuracy largely depends on the commitment and dedication of collaborating physicians. Our database confirms that FA severely impairs the survival of affected patients; the median survival observed in our series (about 22 years) was slightly lower than those reported by the International Fanconi Anemia Registry[16] and by the USA National Cancer Institute.[24] In our Registry, we have not systematically investigated any genotype-phenotype correlation;[25] however, genetic data from a subset of patients (as well as independent data on the genetics of FA in the same geographic area)[26] seem predominantly influenced by the large prevalence of patients harboring *FANCA* mutations.[21,27] Malignancies play an important role in the natural history of FA, the risk increasing with age for a wide array of cancer types;[10-12,18] moreover, some patients can develop multiple cancers, possibly also due to the increased risk associated with anti-cancer treatments (i.e., chemotherapy and radiotherapy). This report confirms the cancer propensity of FA patients and further stresses the need for frequent and careful tumor evaluations, aiming at early therapeutic interventions,[24,28] the only effective strategy for improving long-term survival in FA patients.

Our Registry was not designed to formally investigate the impact of specific therapeutic interventions on the natural history of the disease. However, even if a head-to-head comparison is impossible, we have separately looked for overall survival in FA patients who have received an HSCT, without showing any difference with non-transplanted patients. HSCT may cure the hematological disease associated with FA, but it does not reverse the phenotype that results from the involvement of extra-hematological tissues and organs.[29-31] This is especially true for the intrinsic risk of cancer due to the genetic instability typical of FA, which might actually be increased by the pre-transplant conditioning regimen and possible detrimental

Table 3. Disease manifestations during the whole disease course.

Variable	180 Patients (%)
Hematological disease	
BMF	
no	8 (4)
yes	172 (96)
Hematological malignancies	
no	166 (92)
yes	14 (8)
MDS	9 (5)
AML	4 (2.5)
Lymphoma	1 (0.5)
Solid tumors	
no	160 (89)
yes	20 (11)
Sites of solid tumors°	
head/neck	12 (7)
liver	3 (2)
breast	2 (1)
thyroid	2 (1)
genital tract	2 (1)
lung	1 (0.5)
central nervous system	1 (0.5)
kidney	1 (0.5)
soft tissues	1 (0.5)
skin	1 (0.5)
gastrointestinal tract	1 (0.5)
HSCT	
no	78 (43.5)
yes	102 (56.5)
Sibling	39* (22)
MUD	49§ (27)
CBU	4 (2)
Haploidentical	1 (0.5)
Unknown	9 (5)
Alive	
yes	94 (52)
no	86 (48)

°The total number of tumors exceeds the number of patients with cancer, since some patients experienced multiple tumors: one patient had 4 tumors (genital tract, breast, skin and head/neck), 4 patients had 2 tumors (head/neck and gastrointestinal tract, head/neck and liver, head/neck and thyroid, central nervous system and Wilms). *2 from sibling cord blood units. §One was a second HSCT after a graft failure of a first HSCT from a sibling donor

effects of GvHD.[28,32] Notably, in our study the risk of solid tumors remains high even after HSCT, but apparently it is not increased over that of non-transplanted patients. However, these data should be confirmed with a longer follow-up, possibly within International studies designed to specifically investigate this endpoint. Our observation that the survival of transplanted and non-transplanted patients was not different is not surprising, because HSCT in the context of FA carries specific challenges. Beyond the fact that HSCT does not affect the extra-hematological phenotype of FA, other reasons may play a role: i. HSCT patients may be biased toward a more severe phenotype; ii. initial patients may have received a HSCT with a non-optimized conditioning regimen;[33] iii. initial patients have received HSCT quite late in their disease course; and iv. longer follow-up is needed to let the positive impact of HSCT emerge. Unfortunately, even if in recent decades improvements in transplant procedures (e.g., the use of reduced intensity conditioning regimens) have significantly prolonged the overall survival rate of patients,[31] HSCT for FA remains associated with a poor prognosis, with a high number of patients exposed to lethal complications. Since the RIAF was not designed to study HSCT in the context of FA, the actual impact of HSCT on the natural history of FA needs to be investigated in more specific studies that also deal with all the transplant-specific factors affecting the outcome of HSCT. Indeed, the question is whether more recent HSCT, performed according to

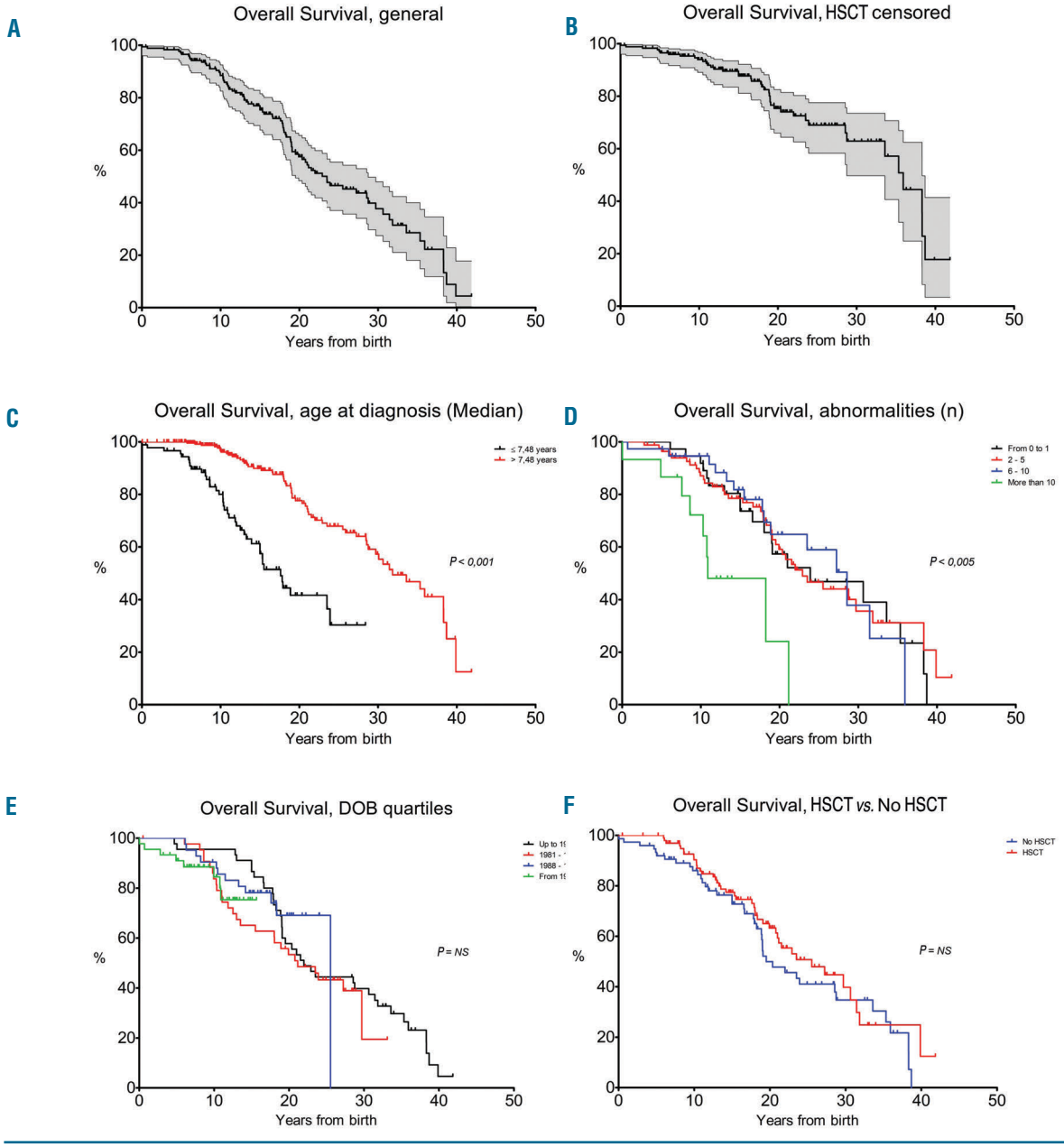


Figure 2. Overall survival. (A) Overall survival, HSCT not censored (filled area represents 95% confidence interval); (B) Overall survival, HSCT censored (filled area represents 95% confidence interval); (C) Overall survival, according to age at diagnosis (patients were grouped based on the median age at diagnosis of 7,48 years); (D) Overall survival, according to number of structural abnormalities; (E) Overall survival, according to DOB quartiles (HSCT not censored); (F) Overall survival: HSCT *vs.* no HSCT.

Table 4. Multivariate analysis.

Variable	HR	95% CI (lower)	95% CI (upper)	P
Gender	1,067	0,643	1,772	0,802
Date of birth (quartiles)	-	-	-	0,305
Age at diagnosis	**0,873**	**0,821**	**0,928**	**<0,001**
Congenital abnormalities (n>10, except skin)	**6,504**	**1,406**	**30,086**	**0,017**
Skin hyper- hypopigmentation	0,665	0,394	1,125	0,128
Skeletal abnormalities	1,093	0,548	2,18	0,8
CNS and/or eyes abnormalities	0,84	0,377	1,873	0,67
Renal and urinary tract abnormalities	1,163	0,589	2,298	0,663
Genital tract abnormalities	0,778	0,365	1,659	0,516
Gastrointestinal tract abnormalities	1,523	0,677	3,426	0,31
Endocrine abnormalities	0,619	0,246	1,563	0,311
Cardiovascular abnormalities	0,399	0,129	1,234	0,111
BMF at diagnosis	1,846	0,757	4,504	0,178
BMF anytime	0,823	0,171	3,964	0,808
Hematological malignancies	1,7	0,813	3,558	0,159
Solid tumours	0,595	0,294	1,204	0,149
HSCT	0,724	0,403	1,301	0,28

transplant protocols which have been optimized over the past years,[34] have improved the outcome of FA, as compared with natural history. One may anticipate that combining earlier therapeutic intervention with improved HSCT protocols may lead in the near future to improved long-term outcomes for FA patients,[35,36] especially if a lack of an increased risk of malignancies is confirmed.

In conclusion, our registry confirms the adverse natural history of FA, eventually leading to disappointing outcomes that have not improved over time; thus, there is an urgent need for effective treatment strategies. Our findings highlight that large collaborative studies are essential to investigate the impact of available therapeutic interventions (such as transplantation), to optimize their use and to define their role in the treatment algorithm of FA. It seems obvious that it will only be through stronger collaboration among physicians and scientists, National and International Registries, and healthcare networks, that we may hope to offer better long-term outcomes to patients affected by FA and to their families.

Table 5. Causes of death.

Cause	No HSCT (%)	HSCT (%)	Total (%)
Infections	14 (33)	11 (25.5)	25 (29)
Bleeding	8 (18.5)	0 (0)	8 (9.5)
Tumor	9 (21)	4 (9)	13 (15)
Liver failure	1 (2)	0 (0)	1 (1)
Kidney failure	2 (5)	0 (0)	2 (2.5)
Heart failure	1 (2)	0 (0)	1 (1)
GvHD	0 (0)	11 (25.5)	11(13)
Other TRM	0 (0)	13 (30)	13 (15)
Other	0 (0)	1 (2)	1 (1)
Unknown	8 (18.5)	3 (7)	11(13)
	43	43	86

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Appendix

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Nonmyeloablative Peripheral Blood Haploidentical Stem Cell Transplantation for Refractory Severe Aplastic Anemia

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A B S T R A C T

New transplant approaches are urgently needed for patients with refractory severe aplastic anemia (SAA) who lack a matched sibling or unrelated donor (UD) or who have failed UD or cord blood transplant. Patients with refractory SAA are at risk of later clonal evolution to myelodysplastic syndrome and acute leukemia. We report our pilot findings with haploidentical hematopoietic stem cell transplantation (haploHSCT) using uniform reduced-intensity conditioning with postgraft high-dose cyclophosphamide in 8 patients with refractory SAA or patients who rejected a prior UD or cord blood transplant. Six of 8 patients engrafted. Graft failure was associated with donor-directed HLA antibodies, despite intensive pre-HSCT desensitization with plasma exchange and rituximab. There was only 1 case of grade II skin graft-versus-host disease. We show that haploHSCT can successfully rescue refractory SAA patients who lack donor-directed HLA antibodies but not in the presence of donor-directed HLA antibodies. This novel protocol for haploHSCT for SAA has been adopted by the European Group for Blood and Marrow Transplantation Severe Aplastic Anaemia Working Party for a future noninterventional, observational study to further evaluate its efficacy.

INTRODUCTION

Patients with severe aplastic anemia (SAA) who lack a matched sibling or unrelated donor (UD) and who fail to respond to immunosuppressive therapy are at increased risk of death from infection and hemorrhage and later clonal evolution to myelodysplastic syndrome and acute myeloid leukemia. Risk factors for myelodysplastic syndrome/acute myeloid leukemia include older age, short telomeres, presence of monosomy 7, prolonged use of granulocyte colony-stimulating factor (G-CSF), and multiple courses of immunosuppressive therapy [1-5]. Hence, there is a need to explore alternative donor hematopoietic stem cell transplantation (HSCT) using either a haploidentical family donor [6-12] or cord blood [13,14], because this is a potentially curative option. However, most studies report poor outcomes, due to poor engraftment, and high risk of graft-versus-host disease (GVHD) with haploidentical HSCT (haploHSCT). Attractions of haploHSCT are graft availability for most patients, less expensive procedural cost than cord HSCT, and shorter time to procurement of the graft. However, published data are limited and mostly restricted to children and young adults [7-11].

Post-transplant cyclophosphamide (CY) has been used in haploHSCT for hematological malignancies to selectively deplete donor alloreactive T cells and thereby reduce acute GVHD [15,16]. In the setting of aplastic anemia, high-dose CY without stem cell support can salvage a significant proportion of refractory patients, but this approach has not been met with widespread enthusiasm because of prolonged pancytopenia after therapy [17]. A nonmyeloablative regimen with postgraft CY was reported in 2 patients with hemolytic paroxysmal nocturnal hemoglobinuria and 1 with both paroxysmal nocturnal hemoglobinuria and sickle cell disease, resulting in sustained engraftment and absence of GVHD in 2 patients [11]. In 2011, Dezern et al. [18] reported the first use of post-transplant CY for GVHD prophylaxis in 2 high-risk SAA patients. They used matched sibling donors and myeloablative conditioning, with both patients engrafting and no GVHD [18]. The Hopkins group also reported 17 cases using the mini-haploidentical approach with post-transplant CY in sickle cell disease. There were no cases of mortality or GVHD, but primary graft failure was a problem [19].

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In this study we used a uniform conditioning regimen using reduced-intensity conditioning with post-transplant CY for haploHSCT in 8 SAA patients, 4 with refractory SAA and 4 after failed UD or cord blood HSCT. We also used G-CSF mobilized peripheral blood stem cells (PBSCs) instead of bone marrow (BM) to achieve a high dose of infused $CD34^+$ cells for engraftment.

METHODS

Eight patients were transplanted, 4 had refractory acquired SAA and 4 failed to engraft after UD (n = 3) or cord blood (n = 1) HSCT. Of the 3 patients who had failed previous UD HSCT, 2 had failed 2 previous UD transplants. Six of 8 patients had acquired idiopathic SAA. Two patients had secondary severe marrow aplasia, 1 after chemotherapy for Hodgkin disease and who subsequently failed UD HSCT and 1 who failed 2 UD HSCTs for myelodysplastic syndrome. Median age was 32 years (range, 19 to 57) and median disease duration 46 months (range, 6 to 91). Family donors were siblings (n = 5), parents (n = 2), and children (n = 1), and HLA matching was 5/10 (n = 6) and 6/10 (n = 2). Five patients were included in the previously reported multicenter series [20].

Conditioning regimen was fludarabine 30 mg/m^2 (days −6 to −2), CY 14.5 mg/kg (days −6 and −5), and total body irradiation 2 Gy (day −1). Unmanipulated G-CSF–mobilized PBSCs were infused at a median $CD34^+$ cell dose of 6. 2×10^6/kg (range, 1.8 to 8.3). GVHD prophylaxis was CY 50 mg/kg/d (days +3 and +4), tacrolimus for 9 months maintaining trough drug at 10 to 15 μg/L with tapering between 9 and 12 months, and mycophenolate 15 mg/kg until day +35.

Median follow-up of survivors was 14.8 months (range, 7.2 to 44.4). Median Karnofsky and HCT-comorbidity index scores were 80% (range, 50 to 90) and 3 (range, 0 to 5), respectively. Neutrophil and platelet engraftment and acute and chronic GVHD were defined as previously reported [21]. Chimerism was assessed in unfractionated BM and peripheral blood $CD3^+$ T cells and $CD15^+$ granulocyte populations. Full donor chimerism was defined as >95% donor hematopoietic cells and mixed chimerism as 5% to 95% cells [21,22]. All patients were screened for cytomegalovirus, Epstein-Barr virus, and adenovirus at twice weekly intervals for the first 3 months. HLA antibodies were measured routinely pretransplant in all patients using microbead flow cytometry assay with mean fluorescence intensity read-out for serum antibody level.

RESULTS

Six patients had sustained neutrophil engraftment; median time to neutrophil engraftment was 18.5 days (range, 16 to 23), and 5 sustained platelet engraftment with a median time of 26 days (range, 21 to 27) (Table 1). Full donor chimerism in unfractionated cells and CD3 and CD15 lineages was achieved and maintained at last follow-up (Table 2). These results are different from the pattern of chimerism as previously reported from our group using fludarabine, CY, alemtuzumab (FCC) conditioning regimen for SAA patients transplanted from sibling or matched UDs, where a high incidence of mixed T cell chimerism in the presence of full donor myeloid chimerism was observed, associated with a very low incidence of chronic GVHD [22]. We therefore compared BM trephine cellularity at days +28 and +100 and at 1 year after haploHSCT with results using FCC conditioning for sibling and UD HSCT from our center. As seen in Supplementary Figure 1, the median BM cellularity at each time point was not significantly different between the 2 groups.

Two patients (patients 6 and 8) who failed to engraft died on days +60 and +137 from sepsis. Both had multiple HLA antibodies directed against the donor which persisted at high level despite treatment with rituximab (375 mg/m^2 weekly for 4 weeks) and plasma exchanges pretransplant (Figure 1A,B). In contrast, sustained engraftment occurred in patients with no HLA antibodies. For patient 6, there was a reduction in the HLA-B51, donor-directed, antibody level, but despite this the patient failed to engraft. The second patient with multiple donor-directed HLA antibodies achieved a significant reduction in antibody levels for 3 antibodies but not for HLA-DR53 specific antibody.

Cytomegalovirus and Epstein-Barr virus viremia occurred in 2 and 5 patients, respectively, but no cases of cytomegalovirus disease or Epstein-Barr virus post-transplant lymphoproliferative disorder occurred. There was no adenovirus viremia. One patient developed Guillain-Barré syndrome (patient 2) with no viral pathogens identified. No response to intravenous immunoglobulin occurred, but some improvement was found with plasmapheresis. During this episode the patient required a prolonged period of intubation during a 92-day intensive care unit admission, which was complicated by recurrent respiratory infections with multiple organisms. At the time of death he was independent of platelet transfusions with the use of eltrombopag and required RBC transfusion approximately 6 weekly.

There was only 1 case of acute GVHD (grade II skin) and no chronic GVHD; however, follow-up was short, with a median follow-up of patients of 12.2 months (range, 3.2 to 40.4). The conditioning regimen was well tolerated, with no hemorrhagic cystitis. One patient (patient 3) delivered a healthy baby at term on day +560 post-transplant. The observation that this regimen does not appear to cause gonadal failure is supported by a previous report of 2 successful pregnancies using this regimen [23].

DISCUSSION

We show in a small cohort of patients with aplastic anemia that haploHSCT using a uniform, nonmyeloablative conditioning regimen with post-transplant CY and with PBSCs as stem cell source rather than BM is a feasible option. Remarkably, the procedure was able to rescue all 4 patients who had primary graft failure after a matched UD transplant or cord blood HSCT, including 2 patients with nonengraftment after 2 unrelated grafts. Furthermore, our patients had a high HCT-comorbidity index and poor performance status. This series includes 2 patients with secondary aplasia, 1 due to chemotherapy for Hodgkin lymphoma. Although the mechanism underlying the marrow failure in these 2 cases is different from those with idiopathic SAA, these cases also illustrate the efficacy of this approach after failed matched UD or cord blood donor HSCT in the presence of severe pancytopenia and a hypocellular BM.

In contrast to our previous finding of mixed T cell chimerism with sustained myeloid engraftment using alemtuzumab-based conditioning for matched sibling and UD HSCT for SAA, in this study we observed sustained donor T cell and myeloid engraftment in all assessable patients. We used G-CSF–mobilized stem cells in preference to BM cells to optimize the infused stem cell dose for halpoHSCT. Stem cell dose is especially important in SAA, where there is an inverse correlation between stem cell dose and graft rejection [24]. The concern with PBSCs of an increased risk of GVHD was not borne out in our small series. We previously reported a high incidence of mixed T cell chimerism with sustained myeloid engraftment and a low incidence of chronic GVHD in SAA patients transplanted from matched sibling donors or UDs using the FCC conditioning regimen [22]. In contrast, in this study of haploHSCT, we showed full donor T cell and myeloid chimerism in engrafted patients. We therefore were interested to know if hematopoietic recovery was associated with a higher degree of BM cellularity after haploHSCT compared with FCC HSCT. However, we showed no significant difference between the 2 groups, but the patient numbers in the

Table 1
Patient Characteristics and Outcomes

Patient No., Age (yr), Gender/Donor	Disease and Severity	Previous Therapy for Aplasia Pre-HaploHSCT	Previous HSCT and Outcome	HCT-CI/ Karnofsky Score	CD34 Stem Cell Dose ($\times 10^6$/kg)	Engraftment (Neutrophil and Platelet)	GVHD (Acute and Chronic)	Median Follow-Up (mo)/Survival Status
1, 19, F/mother	SAA-MDS	ATG + CSA × 2, NR ATG + MMF, PR, relapse MMF-NR	Double cord-GF	0/80	6.7	Day +18 Day +21	None	40.4/alive
2, 51, M/sister	Therapy-related severe aplasia for Hodgkin disease	ATG + CSA, NR	MUD-GF	3/70	4.5	Day +18 No platelet engraftment	None	22/dead Sepsis
3, 23, F/sister	SAA/hPNH	ATG + CSA, PR, relapse	None	3/80	5.8	Day +16 Day +26	Grade II acute GVHD Skin	12.2/alive
4, 57, F/brother	SAA/hPNH	ATG + CSA, CR, relapse, oxymetholone, eculizumab eltrombopag	MUD-GF MUD-GF and Epstein-Barr virus PTLD	5/50	4.9	Day +20 Day +27	None	9.3/alive
5, 22, M/brother	VSAA	ATG + CSA, NR	None	1/90	6.9	Day +23 Day +27	None	3.2/alive
6, 20, M/father	VSAA	CSA, NR ATG + MMF, NR oxymetholone, NR danazol, NR	None	1/90	6.7	Non-engraftment HLA antibody positive	None	2.1/dead Graft failure/sepsis
7, 50, M/son	Therapy-related severe aplasia for MDS-RCMD	None	MUD-GF MUD-GF	4/50	8.3	Day +19 Day +25 100%	None	33.4/alive
8, 41, M/mother	VSAA/PNH	ATG + CSA × 2, CR, relapse × 2, oxymetholone, PR, relapse, eculizumab	None	5/80	1.8	Nonengraftment HLA antibody positive	None	3.1/dead

HCT-CI indicates HCT comorbidity index; MDS, myelodysplastic syndrome; ATG, antithymocyte globulin; CSA, cyclosporine A; NR, no response; MMF, mycophenolate; PR, partial response; GF, graft failure; MUD, matched unrelated donor; hPNH, hemolytic paroxysmal nocturnal hemoglobinuria; PTLD, post-transplant lymphoproliferative disease; VSAA, very severe aplastic anemia; RCMD, refractory cytopenia with multilineage dysplasia; CR, complete response.

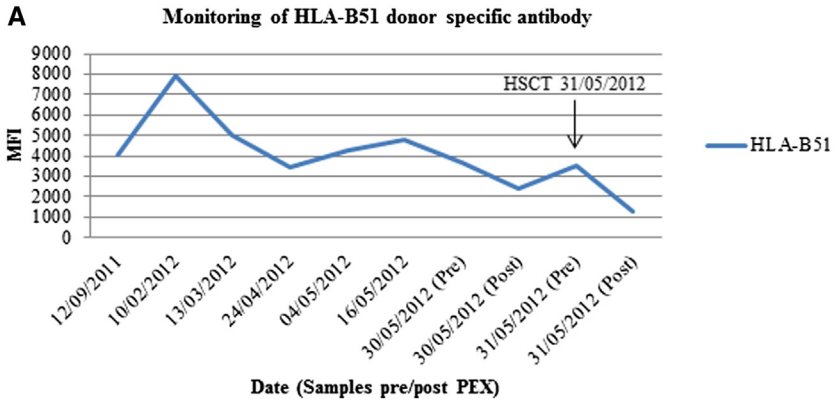
Table 2
Results of Chimerism

Days Post-Transplant	No. Patients Chimerism Tested (Total Assessable)	UF	(Range)	CD3	(Range)	CD15	(Range)
28	6 (8)	100	(99-100)	100	(100-100)	100	(98-100)
56	6 (7)	99	(98-100)	100	(99-100)	99	(98-100)
100	6 (6)	100	(99-100)	100	(99-100)	100	(99-100)
180	5 (6)	100	(96-100)	99	(96-99)	100	(96-100)
365	4 (4)	100	(99-100)	100	(99-100)	100	(100-100)

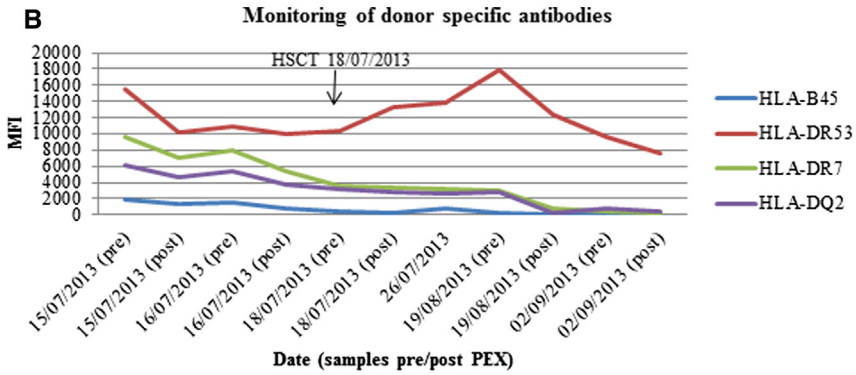
UF indicates unfractionated.
Values in the last 3 columns are median percentages.

haploHSCT group were small, and more patients need to be evaluated.

For both haplo and cord blood HSCT, the presence of recipient HLA antibodies directed against the donor is associated with a high risk of graft rejection [15,16]. Most studies of haploHSCT for SAA do not report routine assessment of donor-directed HLA antibodies. High levels of donor-directed anti-HLA alloantibodies most likely explain the nonengraftment in 2 patients. Intensive desensitization with plasma exchanges and rituximab was insufficient to deplete the



HLA Type	HLA-A	HLA-B	HLA-Cw	HLA-DRB1	HLA-DQB1
Recipient	01:01/29:02	58:01/ 55:01	07:01/ 01:02	03:01/ 14:04	05:03/02:01
Donor	31:01/29:01	58:01/ 51:01	07:01/ 14:02	13:01/ 14:04	05:03/06:03



HLA Type	HLA-A	HLA-B	HLA-Cw	HLA-DRB1	HLA-DQB1
Recipient	23:01;30:01	41:01;42:02	17:01;08:02	11:01	06:02;03:19
Donor	02:01;23:01	45:01;41:01	08:02;41:01	11:01;07:01	06:02;02:02

Figure 1. (A) Patient 6. Changes in HLA antibody levels as measured by the mean fluorescence index (MFI). Recipient and donor HLA typing are shown in the table at the bottom. Before HSCT, this patient had multiple class I HLA antibodies, of which only HLA-B51 was donor directed. After intensive plasma exchanges and rituximab, the antibody level fell from a peak of 7946 MFI to 1304 immediately pre-HSCT. PEX indicates plasma exchange. (B) Patient 8. Changes in HLA antibody levels as measured by MFI. Recipient and donor HLA typing are shown in table at the bottom. Pretransplant, this patient had class I and II HLA antibodies, all of which were donor directed: HLA-B45, -DR7, -DQ2, and -DR53. (HLA-DR53 is directed against a protein encoded by the *DRB4* gene, which is in a haplotype with the DRB1*07:01.) After plasma exchanges and before HSCT, there was a further rise in the HLA-DR53 antibody, likely due to granulocyte transfusions given for invasive fungal infection.

antibodies sufficiently to prevent graft rejection. Despite universal leucodepletion of blood products, HLA alloimmunization remains a problem in up to 30% of patients with aplastic anemia [25,26]. In the setting of HLA matched sibling HSCT, this is due to minor histocompatibility antigens on leucocytes and red cell, and in mismatched HSCT, due to HLA antigens on leucocytes and platelets [27]. An HLA antibody level of 1500 mean fluorescence intensity is associated with graft rejection in the setting of T cell–depleted haploHSCT and >2500 in the setting of T cell–depleted matched UD HSCT due to anti-DPB1 HLA antibodies [28,29]. In our series, for HLA-B51 and HLA-DR53, graft rejection occurred at levels of 1304 and 7621, respectively. Other factors contributing to graft rejection in patient 8 were older donor age (mother's age of 68 years) [30], a low stem cell dose (1.8×10^6 CD34 cells/kg), and a female multiparous donor [28]. It may be that transplanting a much higher CD34 dose would aid engraftment in such cases, but this would potentially increase the risk of acute and chronic GVHD. Thus, for future cases, we agree that routine screening for HLA antibodies is essential pretransplant and, if present, as has been recommended for patients with hematological malignancies, an alternative donor lacking HLA antigen(s) against which recipient HLA antibody (ies) are directed should be used [16]. PBSCs have also recently been used for reduced-intensity haploHSCT with postgraft CY in the setting of hematological malignancy with no adverse impact on GVHD or survival compared with BM [20,31].

In our study of SAA we showed that haploHSCT using postgraft high-dose CY and PBSCs results in sustained engraftment and with minimal GVHD for those patients who lack donor-directed HLA antibodies. However, the presence of donor-directed HLA antibodies was associated with primary engraftment failure, despite attempts to remove the antibodies pretransplant with rituximab and plasma exchange. Because SAA patients have a particularly high risk of HLA alloimmunization, it is essential that all patients are screened pre-HSCT before considering such an approach, because the presence of donor-directed HLA antibodies should preclude the use of that donor.

We propose that this approach to haploHSCT now warrants further exploration in a larger cohort of SAA patients. The European Group for Blood and Marrow Transplantation Severe Aplastic Anaemia Working Party has adopted this protocol for a future noninterventional, observational prospective study of haploHSCT throughout Europe.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbmt.2014.06.028.

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