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"Disorders of Endocrine, Reproductive and Skeletal Systems Following Allogeneic or Autologous Hematopoietic Stem Cell Transplantation for Hematological Malignancies in Adults"

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INTRODUCTION

The stem cell transplantation (SCT) program started 40 years ago as a highly experimental procedure and has progressively become a common treatment for a wide variety of diseases. In the beginning, severe aplastic anemia was the main indication for allogeneic SCT (allo-SCT). Currently, poor prognosis leukemia, other malignant and non malignant hematological disorders, together with advanced or relapsed solid cancers are treated by allo-SCT (1). Today, between 30,000 and 40,000 transplants are performed world-wide per year (2). Underlying diseases leading to allo-SCT are mostly acute or chronic leukemia and lymphoma.

Autologous hemopoietic SCT is performed more frequently than any other type of allogeneic organ transplant world-wide. Underlying diseases leading to autologous stem cell transplant are mostly lymphoma, leukemia and multiple myeloma.

As the number of long-term survivors after SCT is progressively increasing, more attention is now focused on early and late complications of this procedure which may worsen the quality of life and threaten the long-term outcome of transplant recipients (1).

The majority of the data available on the late effects of SCT on the endocrine system concerns the pediatric population, whereas the studies in patients transplanted in adulthood are still scarce. The relative risk of endocrine complications was found to be influenced by the underlying diseases, previous therapies, the use of radiation treatment and the type of its schedule, post-transplant treatments and the age at SCT (3-5). Conditioning regimens that preceed SCT are designed to eradicate the underlying hematological disease (mostly a malignancy) and suppress the host immune system in the allogeneic setting. They consist of highly aggressive antiblastic treatments with or without total body irradiation (TBI). Most of the data concerning the endocrine function derive from the study of patients who underwent TBI as a part of pre-transplantation treatments. TBI has been reported as being responsible for a large part of post-transplant endocrinopathies. Only a few studies have been carried out in adults treated without TBI (6,7); consequently, the impact of chemotherapy alone and/or other possible risk factors in the functioning of the endocrine system is still not completely elucidated in SCT recipients.

The relationship between the endocrine and the immune system is well known; various endocrine glands are target organs that are frequently influenced by dysregulations of the immune system. As a matter of fact, large amounts of cytokines are released at the time of transplantation (8), and immune deficiency occurs because of delayed and incomplete recovery of the immune system, particularly as a consequence of allo-SCT. Graft-versus-host disease (GVHD) simultaneously causes both stimulation and suppression of the immune system, the latter one due to either direct immunosuppressive effects or as a consequence of the treatments of this disorder (9). The prevention and treatment of GVHD consist of high-dose immunosuppressive therapies including mostly glucocorticoids, methotrexate, cyclosporine A and mofetile mycophanolate. Consequently, the integrity of several systems and tissues, including the endocrine, reproductive and skeletal system function, may be affected by multiple exogenous and endogenous damaging factors.

When concerning with bone loss consequent to allo-SCT, many authors have described it as being more persistent and severe at cortical bone (femoral neck) when compared to trabecular sites (lumbar spine) (1-7). Damaging effects on bone mass were attributed to hypogonadism, immunosuppressive and myeloablative treatments, immobilization, increased cytokine release and dietary and metabolic changes concerning with calcium and vitamin D (5,6). One of the most important risk factors for bone loss is represented by precocious ovarian failure (POF), which is also the most common complication of allo-SCT (8-10). Calcium and vitamin D supplementation, and hypogonadism replacement are commonly used in clinical practice after SCT, while bisphosphonates are added only in patients with osteoporosis. Until today, little data is available on the effects of preventive or therapeutic interventions on post-transplant bone loss (1,11-13). No systematic evaluation has been performed yet in comparing the effects of different agents for prevention or treatment of osteoporosis consequent to SCT. All commonly employed antireabsorptive treatments have been shown to improve bone mineral density (BMD) and decrease the rate of fractures in post-menopausal and glucocorticoid-induced osteoporosis (14-17). However, the use of these treatments in transplanted patients may be limited by the general health conditions and by the simultaneous presence of other complications, including acute and chronic GVHD. The liver, skin and gastrointestinal tract are the most common localizations of cGVHD (18), which in the active phase may represent a contraindication for the use of oral or trans-dermal therapies; even a mild chronic GVHD may reduce gastrointestinal or dermal drug absorption. Furthermore, allo-SCT patients usually run into long-lasting and complex

therapeutic regimens in order to prevent or treat other complications; thus, the management of osteoporosis can be difficult in some of them.

Endocrine consequences of the allogeneic stem cell transplantation in adults

The hypothalamus-pituitary-gonadal (HPG) axis

Women

Gonadal failure is the most frequent endocrine consequence of high dose chemotherapy and radiotherapy for any malignancy; recovery is rare and age-dependent, and it is more frequent in girls than in adult women (10,11). Loss of ovarian function occurs as a result of cytotoxic agents and TBI used before transplantation in a dose-dependent manner (6, 12-15). In fact, the LD_{50} (the radiation dose causing the death of 50% of cells) for the human oocyte has been estimated to be less than 4 Gy (16). Sanders *et al.* (14) studied 144 women transplanted for leukemia after conditioning with cyclophosphamide and TBI; they reported hypergonadotropic amenorrhea in 100% of the women for the first years after SCT, and only 6% experienced late ovarian function recovery. All alkylating agents have toxic effects on the ovaries (17), they damage oocytes and supporting granulosa cells of both proliferating and resting follicles in a dose-dependent way (18). The older you get towards the transplant, the lesser the probability of ovarian function recovery; it decreasess by a factor of 0.8 per year of age (14).

In women receiving chemotherapy only, single agent cyclophosphamide was associated with ovarian function recovery in 31% (6 out of 16 women) of patients transplanted < 26 years, but in none of those over 26 yrs (14). The addition of busulphan to cyclophosphamide in either "little" BU/CY (BU 16mg/kg, CY 120 mg/kg) or "big" BU/CY (BU 16 mg/kg, CY 200 mg/kg) regimens caused permanent ovarian damage in everyone apart from some exceptions (19,20).

In a study by Chatterjee et al. (21), acute effects on ovarian function of high dose multiagent chemotherapy did not differ if they were administred with or without TBI. The severity of the acute damage did not predict the probability of the late recovery of ovarian cycles.

In our experience, all but two women had ovarian insufficiency following SCT. About one third of the women became menopausal several months before allografting consequently to previous chemotherapies, while in the remaining patients, the cycles disappeared after conditioning regimens (22). The youngest patient, 13 yr at SCT, and another treated for aplastic anemia (without previous antiblastic agents) experienced a spontaneous recovery of ovarian function with regular menstrual cycles after 12 and 18 months of amenorrhea.

Premature menopause has serious psychological and medical effects (23) and requires treatment. Hormonal replacement therapy (HRT) should be initiated after complete hematological reconstitution but can be contraindicated for long periods in women with severe chronic liver GVHD. In fact, estroprogestins may induce liver toxicity, and they interfere with the hemostatic balance. Moreover, HRT may not be fully effective because of reduced intestinal or cutaneous absorption, as found in 30% of women in our cohort (22). During secondary hypergonadotropic amenorrhea, PRL levels are usually normal and 17-β-estradiol is often undetectable. We have also found reduced androgen circulating levels, likely due to either ovarian damage or adrenal suppression by immunosuppressive treatments of cGVHD (24). In particular, ovarian contribution to lower serum androgens was suggested by the correlation between ovarian volume and patients' estradiol and androgen levels; however, lower DHEAS values in patients with cGVHD were related to longer and more recent corticosteroid use. The impact of a low residual androgen steroid secretion after allo-transplantation on women's health, especially in terms of osteoporosis, cardiovascular risks and quality of life remain to be established as well as any future potential therapeutic implications.

A serious complication in the peri-transplantation period is the polymenorrhea, which can be dangerous due to a suppressed hematopoiesis and the difficulty in controlling the bleeding. The treatment most commonly used is norethisterone acetate which has been shown to be the major risk factor for liver veno-occlusive disease (25). Recently, the use of gonadotropin releasing hormone analogues (aGnRH) with suppressive effects on the hypothalamic-pituitary-gonadal axis, has been shown to prevent peri-transplant vaginal bleeding,; moreover, they do not interfere with the hemostatic balance or cause liver toxicity (26). However, aGnRH administration should be started one month before the conditioning regimen (27) because of initial mild stimulatory effects on the HPG axis. While it has been established that hypogondadism induced by GnRH analogues administration prevent peri-transplant bleeding, there is no sufficient evidence that it can be effective in preventing ovarian damage related to the antiblastic treatments.

Men

Germinal epithelium of the testis is more vulnerable to the effects of chemotherapy and irradiation than the Leydig cells (28) in both childhood and adulthood. A return of elevated FSH levels to the normal range has been occasionally observed in patients treated by high-dose chemotherapy and a single-fraction TBI of 7.5 Gy. This observation has been explained by a recovery of germ-line function (29). Conversely, a very rare recovery of spermatogenesis has been described in adults after fractionated TBI (30). Molassiotis *et al.* (31) observed persistently increased FSH and LH levels in the majority of transplanted males, regardless the use of TBI. Concerning the Leydig cell function, normal testosterone levels were found in most of the patients following SCT by Littley *et al.* (30), whereas Chatterjee *et al.* (21) reported a subtle, but persistent Leydig cell dysfunction. More recently, the same group of authors have focused attention on libido and erectile dysfunction in men who had previously been treated by high-dose chemotherapy and TBI conditioning for myeloma; the athors attributed the sexual dysfunction to cavernosal arterial insufficiency (30). Van Basten did not confirm the presence of arterial dysfunction in a population that was only treated by chemotherapy (32) and thus, this seems to be mainly a consequence of TBI.

We have found a persistent increase in FSH values in 47% of men after allo-SCT, suggestive of spermatogenesis damage. However, azoospermia was also found in men with FSH levels within the high-normal range (22). This finding suggests that germinal tissue damage cannot be ruled out by near-normal gonadotropin values, and a spermiogram is always necessary. Keilholtz et al. (12) and Mertens (33) found a similar FSH increase in long term survivors after autologous SCT (up to 92%); in those studies however, the patients had received radiation treatment. In a study by Grigg, cGVHD was associated with lower sperm count in male long-term survivors after busulphan/cyclophosphamide conditioning, suggesting some possible effects of this chronic complication on gonadal status (19). For men, the age during treatment seems to be less important than for women in the development of gonadal failure (4,12). In contrast, the underlying disease, the type of antiblastic drugs and/or the duration of their administration, all affect the likelihood of spermatogenesis damage.

Testosterone production was unaffected in our long-term survivors out of treatments and nobody reported a regression of secondary sexual characteristics. On the other hand, all men evaluated during GVHD had low testosterone levels, likely due to an inhibitory effect of the immunosuppressive treatments on the hypothalamic-pituitary-gonadal axis. In fact,

glucocorticoids are known to suppress GnRH release and consequently the function of the whole axis. Moreover, the adrenal source of androgens is also inhibited by glucocorticoids. The role of CsA treatment for testicular injury cannot be excluded as it is known to damage the testicles in long term treatments (34).

Testosterone replacement is rarely required given the mostly mild and transient nature of sexual steroid decrease in men after SCT. There is one case report describing the failure of preventing TBI-induced testicular germinal cell damage by GnRH analogue administration (35). Currently, the only possibility to achieve a certain conceivement after allo-SCT is by sperm cryopreservation.

Reproductive function

The majority of patients experience gonadal damage after SCT and a consequent infertility. Thus, options to preserve fertility have been searched. Sperms and embryos conceived by *in vitro* fertilization can currently be well cryopreserved, but oocyte banking, in the absence of a male partner, is more difficult and still an experimental procedure (36). Not only the conservation but also the pick up of germinal cells is technically more complex in women. For a satisfactory oocyte pick up, women have to undergo ovarian stimulation for several weeks before starting high-dose chemotherapy, monitoring the follicular growth by ultrasonography. Following these procedures, an aspiration of the follicles should be performed. All of this is often difficult to achieve due to the severity of the underlying malignant disease and the urgency to start antiblastic treatment as soon as possible. Furthermore, women affected by cGVHD can be predisposed to vulvo-vaginal infections, vaginal and even cervical stenosis and dysfigurement of the internal and external genitalia including the perineum. All of these disorders make it difficult for these women to have sexual intercourse, they can suffer from dyspareunia, reproductive failure and difficult birth labor (37-39).

Sporadically, successful pregnancies have been reported in recent decades, especially in women who underwent autografting (40); however, most of the information regarding this issue derived from two large surveys conducted in Europe and the USA (20,41). In each population, partners of male patients had uncomplicated pregnancies and normal children, whereas women after allografting had a high incidence of miscarriage, pre-term labor and low birth weight babies, indicating the difficulty to carry out their pregnancy due to damage of both the ovaries and uterus. The rate in congenital malformation, developmental delay and

malignant disease was just as high in the offspring of SCT recipients as in the general population. Nevertheless, lower pregnancy rates and crude birth rates were revealed among a mixed population of transplanted patients than in the general population and they were worse in women that were previously treated by TBI. Germinal cell damage, endocrine dysfunction and disorders of pelvic vascularization can all contribute to a worse outcome of the reproductive function after SCT (42-44).

In our experience of a total of 106 patients who had undergone allo-SCT in this center, only two pregnancies occurred: one was reported in a wife of a male patient who had received allograft 3 years earlier. One woman delivered two healthy twins 5 years after allo-SCT with hormonally fully assisted pregnancy with oocytes donated by a sister.

On the other hand, some reports have shown evidence of relapse of leukemia during pregnancy. However, it cannot be excluded that the patients have relapsed independently from pregnancy, and it is uncertain whether the natural history of the primary hematological disease can be influenced by pregnancy. Since the relationship between underlying diseases and pregnancy is still unclear, Salooja *et al.* (41) recommended that pregnancy would be delayed for more than 2 years after a SCT, especially in patients with CML. Moreover, they should have a persistently negative Philadelphia chromosome and bcr/abl transcriptase.

The hypothalamus-pituitary-adrenal (HPA) axis

In accordance with a previous hypothesis on relative radio-resistance of adrenal tissue (3), post-allografting secondary adrenal insufficiency due to suppression of the HPA axis, seems to be related to the duration and cumulative dose of corticosteroid treatments received. Only the initial report by Sanders *et al.* (45) had described a high incidence of adrenocortical dysfunction following TBI and SCT, but subsequent studies have found rare evidence of permanent post-transplant cortisol deficiency (22,46). All of the patients who have developed secondary adrenal insufficiency in our center had previously been treated with corticosteroids for ≥ 10 months for chronic GVHD, at a cumulative dose > 10 gr/m^2. Nevertheless, some variable individual sensitivities of the HPA axis to exogenous suppression can be speculated.

Patients with chronic GVHD, in whom corticosteroid treatment is suddenly withdrawn because of the onset of an important infection, are at a high risk in developing an acute adrenal crisis that can further worsen their clinical condition. However, the prevalence of mild (or dynamic) temporal adrenal insufficiency is still likely to be an underestimated condition, therefore, a stimulation test should be performed to exclude any mild degree of the

HPA axis insufficiency. This procedure is still uncommon, unless thoroughly and specifically investigated by endocrinologists. In our experience, all of the patients recovered from adrenal insufficiency after 3 to 18 months.

The hypothalamus-pituitary-growth hormone axis

Results of previous studies on GH secretion in adults concluded that the pituitary gland is less vulnerable to irradiation damage in adulthood than in childhood. In particular, Littley *et al.* (30) found a normal GH peak response to a GH stimulation test, 17-55 months after SCT with TBI conditioning. Kauppila *et al.* (47) found normal IGF-1 levels in all transplanted patients, but 20% of them showed an impaired response to a provocative test following TBI for a SCT (<5 mcg/l with GHRH administration).

When concerning with growth hormone secretion in children, a reduction in growth and GH secretion have previously been described after cranial irradiation. Height loss after TBI alone resulted less important (48,49). On the other hand, discordant results were obtained in children transplanted for leukemia following BU/CY conditioning alone: Sanders (50) reported a significant incidence of growth hormone deficiency, while Wingard *et al.* (51) found similar growth rates after BU/CY and CY/TBI over the first two post-transplanted years. In contrast to these reports, Liesner (52), Giorgiani *et al*. (53), Michel *et al*. (54) Leiper *et al.* (55) and Afify et al. (56) did not find any significant growth impairment up to 5 years after BU/CY and SCT. More data on the impact of BU/CY on growth are still needed to draw any final conclusion. GVHD has also been found to be associated with impaired growth by Sanders *et al.* (57). Glucocorticoids used for the treatment of this complication have acute growth-suppressive effects, however, decreased growth rates were reported also in untreated patients with cGVHD, suggesting that other factors directly related to the presence of cGVHD contributed to their decreased growth (57). The hypothalamus-pituitary-IGF-I axis function has been poorly investigated in adult allo-SCT recipients and our group has studied this condition after BU/CY conditioning alone. In contrast to the previous findings in adults, we have shown that IGF-I levels were lower than the age-reference values in 38% of patients who were affected by cGVHD, whereas IGF-1 resulted reduced in only 7% of subjects who were free of cGVHD. This finding can in part explain the previous observation made by Sanders *et al*. (57); in fact, cGVHD represents a condition of a multiorgan injury and is associated with a lower BMI. It is well-known that IGF-I reduction is associated with chronic diseases accompanied by situations such as starvation or reduced nutrition (58). The hypothesis of a

possible influence of the general health conditions on the GH-IGF status is further supported by results of Adan *et al.* (59), who have further investigated the effects of post-transplant complications on growth, showing that children with no complication have normal growth in contrast to children with any complications who had decreased growth rates (0.5±0.3SD *vs*. –1.6±0.3SD), all of these children were conditioned with chemotherapy alone.

The hypothalamus-pituitary-thyroid axis

The thyroid dysfunction consequent to TBI occurred both in children and adults (4,31) and may be transient (60). It is well known that external irradiation to the neck may lead to abnormal thyroid function and predispose to thyroid neoplasm. The incidence of thyroid dysfunction following fractionated TBI (15-16%)(46,50,61) appears significantly less than that following single-dose TBI (46-48%)(50,62,63). The long-term natural history of irradiation-induced thyroid dysfunction is still unknown as well as the timing and the peak incidence for TBI schedules (4).

After a TBI dose of 10-12 Gy, the typical biochemical finding is a subclinical hypothyroidism characterized by mildly elevated basal TSH levels and a thyroid hormones within the normal range (46,50,60,63) with overt hypothyroidism being a rare finding. As a matter of fact, Al-Fiar *et al.* (64) has observed a trend with an increase in the incidence of thyroid dysfunction associated with increasing TBI doses.

On the other hand, an eightfold increase in the incidence of hyperthyroidism has been found following neck irradiation for Hodgkin's disease (65), being dose-related and affected by latency period.

Chemotherapy-only conditioning regimens (BU/CY) were also implicated in thyroid dysfunction and TSH increase, with an 11% incidence reported by Al-Fair *et al*. (64) *vs.* 16.7% for 12 Gy TBI. Most of the patients were evaluated within 2 years after SCT. Similar frequency was reported by Toubert *et al.* (14%) in a cohort of patients not treated by TBI (6).

The type of transplant may play some role in the development of thyroid disorders. In fact, sub-clinical and overt thyroid function impairment has been more frequently reported after allo-BMT (6,66,67) but less in autologous BMT recipients (12). Kauppila *et al.* (47) have reported exaggerated TSH response to TRH in 35% of patients after a mean of 3.2 years following allo-SCT. Sub-clinical hypothyroidism was revealed late after allo-SCT in our experience (> 5 years after SCT), all but one had normal baseline TSH levels. L-thyroxin replacement treatment was given to both patients.

Although increased frequency of transient thyreotoxicosis has been described early after allografting and immune induced injury has been claimed as the pathogenetic factor (22,66), hypothyroidism can be discovered years after transplant and has been ascribed principally to TBI and busulphan therapy (57,60,68,69). The prevalence of transient sub-clinical hyperthyroidism in our patients (15%) was in line with data from a recent longitudinal study by Kami *et al.* (12.3%)(66). Antibodies toward thyroid were not necessarily present in patients with sub-clinical hyper- or hypothyroidism. We reported at first a biochemical and ultrasonographic evidence of chronic thyroiditis associated with normal thyroid function in allo-BMT recipients, occurring 2 to 10 years after allo-SCT.

Toubert *et al.* (6) have found low T3 syndrome (FT4 and TSH within, and FT3 below their respective ranges of normality) in 48% of patients at 3 months decreased to 19% at 14 months after allo-SCT, while Schulte *et al.* (70) described this condition in 100% of patients at day 14 after SCT, and its persistence to day 28 was associated with a higher probability of fatal outcome. We have found persistence of the low T3 syndrome in patients with chronic extensive GVHD even 12-48 months after BMT, likely due to decreased extra-thyroidal conversion of thyroxin to 3,5,3' triiodothyronin induced by either chronic disease or glucocorticoid treatment (71,72).

An increase in the prevalence of thyroid papillary carcinoma was found in SCT recipients compared to the general population by two major multicenter studies (one European and one American) on late cancer complications (1,73). However, the number of events are few (5/3182 children). The risk of thyroid tumors following TBI seems to be dose- and age-related (65,74). This is in line with a recent experience of Chernobyl (75), suggesting that the thyroid gland is highly sensitive to the radiation effects at a very young age. The latency period between thyroid irradiation and clinical presentation of the tumor can be many years. As there is a body of evidence from animal studies that TSH increase can be a risk factor for thyroid tumorigenesis, the patients with subclinical hypothyroidism should be treated with a substitutive levothyroxin dose in order to normalize TSH levels. Nevertheless, there is not yet any evidence of reducing thyroid cancer risk by longitudinal studies of irradiated patients. The only thyroid malignancy in our population was a case of thyroid lymphoma 8 months after BMT. The pre-existing thyroid nodules were stable over time in all patients during the observational period up to 36 months in terms of size and consistence.

Risk factors for endocrine disorders

The risk factors for gonadal damage are summarized in the **Table 1**. There are some gender difference in gonadal damage and recovery: males are less frequently affected and are more likely to experience gonadal recovery than females. In cases of gonadal function recovery, male gonads tended to recover more quickly than those female (33,43,76). Total dose and modalities of TBI administration seem to play an important role in damaging endocrine system.

Table 1. Risk factors for gonadal damage after allogeneic SCT

	Risk factors
Patients' factors	
Pubertal status	Post-pubertal> pre-pubertal
Age at SCT in women	>30 yrs
Gender	Female > male
Underlying disease, tendency to infiltrate gonads	ALL, lymphoma
Treatments' factors	
Radiotherapy/type	Local, inverted Y Multiple doses
Chemotherapy	Multiple alkylating agents> single agent Alkylating agents > other chemotherapy
Type of transplant	Allogeneic following TBI > autologous Presence of graft-versus host disease

While multiple fractionating of radiation treatment seems to be less dangerous for the thyroid gland than a single dose TBI, gonads injury and GH deficiency are more frequently caused by repeated radiation doses (**Table 2 and 3**).

Table 2. Risk factors for growth hormone deficiency

	Risk factors
Patients' factors	Childhood > adulthood
Treatments' factors	Cranial irradiation > TBI Radiotherapy > chemotherapy Single dose > fractionated radiotherapy Intrathecal chemotherapy

TBI: total body irradiation

Type of transplant seems to be important also for other endocrine dysfunction. In particular, thyroid and adrenal functions, and IGF-1 impairment occur more frequently in patients after allografting than in those who have underwent auto-SCT.

Table 3. Risk factors for thyroid dysfunction after hematopoietic stem cell transplant

Condition	***Risk factors***	***Time since SCT***
Hypothyroidism	Neck irradiation > TBI Single dose TBI > fractionated TBI Allogeneic > autologous SCT Chronic GVHD	Late effect (years after SCT)
Subclinical hyperthyroidism	Allo-SCT	Earlier effect (within 12 months)
Low T3 syndrome	General conditions, nutritional status Immunosuppressive treatments	Variable (days – years after SCT)
Papillary carcinoma	Radio-chemotherapy > chemotherapy Childhood > adulthood	Late effect (years after SCT)

Among allo-SCT patients, those affected by chronic GVHD had more frequent disorders in IGF-1 secretion and in thyroid and adrenal function (22) (**Table 4**). However, thyroid disorders occurred also in 35.7% of our allo-transplanted patients without chronic GVHD. As thyroid is one of the most frequently involved target organs in the autoimmune disorders also in the general population, its more frequent involvement is present also in the population of patients transplanted with SCT. In our experience, men and women were equally affected by chronic GVHD, while cGVHD presence was associated with older age at SCT and significantly higher cumulative doses of corticosteroids and cyclosporine A compared with patients not affected by this complication. Nevertheless, it is difficult to separate clinical effects of immune system damage and immunosuppressive treatments. No significant difference in thyroid, IGF-I and adrenal impairments was found regarding age at SCT in our adult population (22).

Table 4. Occurrence of endocrine disorders according to chronic graft-versus-host-disease (GVHD) in 40 adult patients treated with allo-SCT following little BU/CY conditioning for leukemia (22).

Variable	***GVHD +***	***GVHD –***
Thyroid disorders	14 (53.8%)	5 (35.7%)
Persistent low T3 syndrome[$]	4 (15.4%)	-
Chronic thyroiditis*	9 (34.6%)	4 (28.6%)
Subclinical hyperthyroidism	4 (15.4)	2 (14.2%)
Subclinical hypothyroidism	2 (7.7%)	-
Thyroid lymphoma	1 (3.8%)	-
Adrenal insufficiency	4 (15%)	-
IGF-I impairment	10 (38%)	1 (7.1%)
Total of patients affected	18 (69%)#	4 (28.6%)

The values express number of patients affected and percentage of the subgroup with or without GVHD. [$]: persistent low T3 syndrome means the presence of normal FT3 and FT4 values and TSH levels below the low limit of the normal range at least 12 months after allo-BMT; *: diagnosis of chronic thyroiditis was based on autoantibodies to thyroid presence and typical ultrasound pattern; **: $p < 0.001$ *vs* group without GVHD by Mann-Whitney test; #: $\chi 2 = 6.078$; $p = 0.048$ *vs* group without GVHD.

Conclusions

High prevalence of endocrine dysfunction in allo-SCT recipients have been described world-wide by groups performing SCT. Gonadal failure was found in 95-99% of women and in 60-92% of men, both related prevalently to TBI and alkylating agents administration. On the other hand, thyroid, adrenal and IGF-I impairments were late events occurring in up to 47.5%, 10% and 27% of all patients, respectively, being related to antiblastic treatments, immune system derangement and to immunosuppressive treatments.

Allogeneic SCT has become a successful treatment for different hematological malignancies and other diseases as well, and is generally performed in young patients (1,3,77). Multiple late complications have already been described but few data on risk factors of these long term effects of SCT are available, except for gonadal failure. Endocrine disorders were mostly ascribed to highly aggressive chemotherapy and TBI. However, multiple disorders were found in the endocrine system function of patients not treated by TBI, even years after SCT. In particular, we have recently demonstrated that cGVHD profoundly impairs endocrine functions in allo-STC recipients, even in absence of TBI. While the toxic effects of antiblastic agents have been widely described, the impact of immune system damage on endocrine function is less clear. Allogeneic bone marrow/blood derived stem cell transplantation is the only case of great amount of donor immunocompetent cells infused to a host recognizable as “nonself”, and thus causing graft-versus–tumor reaction, and GVHD. While the first is necessary for disease disappearance, the second represents a frequent complication that requires immunosuppressive treatments further compromising immune system function (78,79). GVHD is divided into acute and chronic disease based upon the time of onset. Skin, liver and gastrointestinal tract are principally affected (80-82); however, more target organs can be involved in the chronic disease and autoantibody formation – usually related to autoimmune disorders-, is more frequently described in the chronic disease (79,81,82). Patients developing cGVHD seem to be at greater risk for endocrine dysfunction and increased frequency of thyroid disorders, adrenal insufficiency and IGF-I. This findings suggest that immunosuppressive treatments and immune system derangement play an important role in the development of endocrine dysfunction after allografting. This hypothesis is further supported by a previous finding of normal thyroid and adrenal function 5 years after autologous bone marrow/blood-derived progenitor cell transplantation (12).

Survival for malignant diseases might have as consequence endocrine failure and this can be even more relevant considering that patients undergoing allo-BMT are younger than recipients of autologous BMT and other organ transplants. Therefore, they require life-long attention to detect, prevent and treat symptoms and disorders of endocrine dysfunction. Furthermore, important fertility problems remain still to be resolved (35-37,83). A multidisciplinary management to patients after allogeneic bone marrow-derived hemopoietic stem cells is mandatory.

References

1. Kolb HJ, Socié G, Duell T, Van Lint MT, Tichelli A, Apperley JF*, et al*. For the late effects working party of the European cooperative group for blood and marrow transplantation and the European late effects project group. Malignant neoplasms in long-term survivors of bone marrow transplantation. *Ann Intern Med* 1999;131:738-44.

2. Horowitz M. Uses and growth of hematopoetic cell transplantation. In: Hematoipoietic Cell Transplantation, 2nd edn (ed by ED Thomas, KG Blume & SJ Forman) Blackwell Oxford 1998, pp 12-18..

3. Shalet SM, Didi M, Oglivy-Stuart AL, Schulga J, Donaldson MDC. Growth and endocrine function after bone marrow transplantation.[Review] *Clin Endocrinol* 1995;42:333-339.

4. Brennan BMD & Shalet SM. Endocrine late effects after bone marrow transplant. [Review]. *Br J Haematol* 2002; 118:58-66.

5. Chatterjee R, Kottaridis PD. Treatment of gonadal damage in recipients of allogeneic or autologous transplantation for haematological malignancies. Mini-review. *Bone Marrow Transplant* 2002; 30:629-635.

6. Toubert ME, Sociè G, Gluckman E, Aractingi S, Espèrou H, Devergie A, *et al.* Short- and long-term follow-up of thyroid dysfunction after allogenic bone marrow transplantation without the use of preparative total body irradiation. *British Journal of Haematology* 1997;98:453-57.

7. Galimberti M, De Sanctis V, Lucarelli G, Polchi P, Angelucci E, Baronciani D, *et al*. Endocrine function after bone marrow transplantation for thalassemia. *Bone Marrow Transplant* 1991;7(Suppl 2):74.

8. Jadus MR & Wepsic MT. The role of cytokines in graft-versus-host reaction and disease. *Bone Marrow Transplant* 1992;10:1-4.

9. Sullivan KM, Agura E, Anasetti C, Appelbaum F, Badger C, Bearman S, *et al*. Chronic graft-versus-host-disease and other late complications of bone marrow transplantation. *Semin Hematol* 1991;28:250-259.

10. Goodwin PJ, Ennis M, Pritchard KI, Trudeau M, Hood N. Risk of menopause during the first year after breast cancer diagnosis. *J Clin Oncol* 1999;17**:** 2365-2370.

11. Apperley JF. Late complications of transplants in adults. Fertility after high dose therapy. In: Blood and Marrow Transplantation (The European School of Haematology Handbook 1998) Eds: J Apperley, E. Gluckman, A. Gratwohl Associate Editor: C. Craddock. Paris 1998, pp 151-154.

12. Keilholtz U, Max R, Scheibenbogen C, Wüster Ch, Körbling M, Haas R. Endocrine function and bone metabolism 5 years after autologous bone marrow/blood-derived progenitor cell transplantation. *Cancer* 1997;79:1617-22.

13. Schimmer AD, Quatermain M, Imrie K, Ali V, McCrae J, Stewart AK, Crump M*, et al*. Ovarian function after autologous bone marrow transplantation*. J Clin Oncology* 1998;16:2359-63.

14. Sanders JE, Buckner CD, Amos D, Levy W, Appelbaum FR, Doney K, *et al*. Ovarian function following marrow transplantation for aplastic anemia or leukemia. *J Clin Oncol* 1988;6:813-18.

15. Apperley JF & Reddy N. Mechanism and management of treatment related gonadal failure in recipients of high-dose chemoradiotharapy. *Blood Review* 1995;9:93-116.

16. Wallace WHB, Shalet SM, Hendry JH, Morris-Jones PH, Gattamaneni HR. Ovarian failure following abdominal irradiation in childhood; natural history and prognosis. *Clin Oncol* 1989;1:75-79.

17. Chabner BA, Allegra CJ, Curt A. Antineoplastic agents. In Hardman JG, Limbird LE (eds): Goodman & Gilman's The Pharmacological Basis of Therapeutics (ed 9). New York, NY, McGraw-Hill, 1996, pp 1233-87.

18. Warne G, Fairley KF, Hobbs JB, Martin FIR. Cyclophosphamide-induced ovarian failure. *N Engl J Med* 1973;289:1159-62.

19. Grigg, A.P., McLachlan, R., Zaja, J., Szer, J. Reproductive status in long-term bone marrow transplant survivors receiving busulphan-cyclophosphamide (120 mg/kg). *Bone Marrow Transplant* 2000; 26, 1089-1095.

20. Sanders JE, Hawley J, Levy W, Gooley T, Buckner CD, Deeg HJ, Dooney K, Storb R, Sullivan KM, Witherspoon RP, Appelbaum FR. Pregnancies following high-dose cyclophosphamide with or without high-dose busulphan or total-body irradiation and bone marrow transplantation. *Blood* 1996;87:3045-3052.

21. Chatterjee R, Mills W, Katz M, McGarrigle HH, Goldstone AH. *Bone Marrow Transplant* 1994;13;511-517.

22. Tauchmanovà L, Selleri C, De Rosa G, Pagano L, Orio F. Jr, Lombardi G, Rotoli B, Colao A. High prevalence of endocrine dysfunction in long-term survivors after allogeneic bone marrow transplantation for hematological diseases. *Cancer* 2002; 95:1076-1084.

23. Chiodi S, Spinelli S, Cohen A, Guaalaandi F, Lamparelli T, Lavagetto A, et al. Cyclic sex hormone replacement therapy in women undergoing allogenic bone marrow transplantation: Aims and results. *Bone Marrow Transplant* 1991;8 (Suppl):47-9.

24. Tauchmanovà L, Selleri C, Esposito M, Di Somma C, Pivonello R, Orio F Jr, Lombardi G, Rotoli B, Colao A. Ovarian hypoplasia and dysfunction in pre-menopausal women after allogeneic or autologous stem cell transplantation for hematological malignancies. The Endocrine Society's 84th Annual Meeting, San Francisco, June 19-23, 2002. Abstract Book P3-514.

25. Hägglund H, Remberger M, Klaesson S, Lönnqvist B, Ljungman P, Rindgden O. Noretisterone treatment, a major risk-factor for veno-occlusive disease in the liver after allogeneic bone marrow transplantation. *Blood* 1998; 92:4568-4572.

26. Laufer MR, Townsend NL, Parsons KE, Brody KA, Diller LR, Emans SJ, Guinan EC. Inducing amenorrhea during bone marrow transplantation. A pilot study of leuprolide acetate. J Reprod Med 1997; 42:537-541.

27. Sica S, Salutari P, Chiusolo P, Cicconi S, Sorra F, Laurenti L, Piccirillo N, Scirpa P, Leone G. Hormonal therapy after stem cell transplantation and the risk of veno-occlusive disease. Letter *Blood* 1999; 93:3154.

28. Shalet SM. Cancer therapy and gonadal dysfunction. In: Clinical Endocrine Oncology (ed. by R. Sheaves, P. Jenkins & JAH Wass) Blackwell Science Ltd, Oxford, UK 1994, pp 510-513.

29. Sklar CA, Kim TH, Ramsay NKC. Testicular function following bone marrow transplantation performed during or after puberty. *Cancer* 1984; 53:1498-1501.

30. Littley MD, Shalet SM, Morgenstern GR, Deakin DP. Endocrine and reproductive dysfunction following fractionated total body irradiation in adults. *Quarterly J Medicine* 1991;287:265-274.

31. Molassiotis A, van den Akker OBA, Milligan DW, Boughton BJ. Gonadal function and psychosexual adjustment in male long-term survivors of bone marrow transplantation. *Bone Marrow Transplant* 1995;16:253-259.

32. Van Basten JP, van Driel MF, Hoekstra HJH, Sleijfer DT. Erectile dysfunction with chemotherapy. Lancet 2000;35: 169.

33. Mertens AC, Ramsy NKC, Kouris S, Neglia JP. Patterns of gonadal dysfunction following bone marrow transplantation. *Bone Marrow Transplant* 1998; 22:345-350.

34. Handelsman DJ, Mc Dowell IF, Caterson ID, Tiller DJ, Hall BM, Turtle JR. Testicualar function after renal transplantation: comparison of Cyclosporin A with azathioprine and prednisone combination regimens. Clin Nephrol 1982;22:144-148.

35. Chatterjee R, Kottaridis PD, McGarrigle HH, Papatryphonos A, Goldstone AH. Hypogonadism fails to prevent severe testicular damage induced by total body irradiation in a patient with beta-thalassaemia major and acute lymphoblastic leukemia. *Bone Marrow Transplant* 2001; 28:989-991.

36. Fabbri R, Porcu E, Marsella T, Rocchetta G, Venturoli S, Flamigni C. Human oocyte cryopresevation: new perspectives regarding oocyte survival. *Human Reproduction* 2001;16:411-416.

37. Schubert MA, Sullivan KM, Schubert MM, *et al.* Gynecological abnormalities following allogenic bone marrow transplantation. *Bone Marrow Transplant* 1990;5:425-430.

38. Anguenot JL, Ibèchèole V, Helg C, Piacenza JM, Dumps P, Bonnefoi H. Vaginal stenosis with hematocolpometra, complicating chronic graft versus host disease. *Europ J Obstet & Gynecol and Reprod Biol* 2002; 103:185-187.

39. Syrjala KL, Roth-Roemer SL, Abrams JR, Scanlan JM, Chapko MK, Visser S, Sanders JE. Prevalence and predictors of sexual dysfunction in long-term survivors of marrow transplantation. *J Clin Oncol* 1998; 16:3148-3157.

40. Herschlag A & Schuster M. Return of fertility after autologous stem cell transplantation. *Fertil Steril* 2002; 77:419-421.

41. Salooja N, Szydlo RM, Sociè G, Rio B, Chatterjee R, Ljungman P, Van Lint MT, Powels R, Jackson G, Hinterberger-Fischer M, Kolb HJ, Apperley JF. Pregnancy outcomes after peripheral blood or bone marrow transplantation: a retrospective survey. *Lancet* 2001; 358: 271-276.

42. Harris E, Mahendra P, McGarrigle HH, Linch DC, Chatterjee R. Gynacomastia with hypergonodotropic hypogonadism and Leydig cell insufficiency in recipients of high-dose chemotherapy or chemo-radiotherapy. *Bone Marrow Transplant* 2001;28:1141-1144.

43. Anserini P, Chiodi S, Spinelli S, Costa M, Conte N, Copello F, Bacigalupo A. Gonadal function post transplantation. Semen analysis following allogeneic bone marrow transplantation. Additional data for evidence based counselling. *Bone Marrow Transplant* 2002; 30:447-451.

44. Chatterjee R, Mills W, Katz M, McGarrigle HH, Goldstone AH. Germ cell failure and Leydig cell insufficiency in post-pubertal males after autologous bone marrow transplantation with BEAM for lymphoma. *Bone Marrow Transplant* 1994;13:519-522.

45. Sanders JE, Pritchard S, Mahoney P, Amos D, Buckner CD, Witherspoon RP, Deeg HJ, Doney KC, Sullivan KM, Appelbaum FR, Storb R, Thomas ED. Growth and development following marrow transplantation for leukemia. *Blood* 1986;68:1129-1135.

46. Ogilvy-Stuart AL, Clark DJ, Wallace WHB, Gibson BE, Stevens RF, Shalet SM, Donaldson MDC. Endocrine deficit after fractionated total body irradiation. *Archives of Diseases of the Child* 1992;67:10.

47. Kaupilla M, Koskinen P, Irjala K, Remes K, Viikari J. *Bone Marrow Transplant* 1998; 22:331-337.

48. Cohen A. Late complications of transplants. Endocrinological complications. In: Blood and Marrow Transplantation (The European School of Haematology Handbook 1998) Eds: J Apperley, E. Gluckman, A. Gratwohl Associate Editor: C. Craddock. Paris 1998, pp 154-159.

49. Holm K, Nysom K, Rasmussen MH, Kertz H, Jacobsen N, Skakkebaek NE, Krabbe S, Muller J. Growth, growth hormone and final height after BMT. Possible recovery of irradiation-induced growth hormone insufficiency. *Bone Marrow Transplant* 1996; 18:163-170.

50. Sanders JE & The Seattle Bone Marrow Transplant Team. The impact of marrow transplant preparative regimens on subsequent growth and development. *Seminars in Hematology* 1991;28:244-249.

51. Wingard JR, Plotnick LP, Freemer CS, Zahurak M, Piantadosi S, Miller DF, Vriesendorp HM, Yeager AM, Santos GW. Growth in children after bone marrow transplantation: busulphan plus cyclophosphamide versus cyclophosphamide plus total body irradiation. *Blood* 1992; 79:1068-1073.

52. Liesner JR, Leipner AD, Hann IM, Chessells JM. Late effects of intensive treatment for acute myeloid leukemia and myelodysplasia in childhood. *J Clin Oncol* 1994;12,917-924.

53. Giorgiani G, Bozzola M, Locatelli F, Picco P, Zecca M, Cisternino M, Dallorso S, Bonetti F, Dini G, Borrone C, Regazzi MB, DeStefano P, Severi F. Role of busulphan and total body irradiation on growth of prepubertal children receiving bone marrow transplantation and results of treatment with recombinant human growth hormone. *Blood* 1995;86: 825-831.

54. Michel G, Sociè G, Gebhard F, Bernaudin F, Thuret I, Vannier JP, *et al. J Clin Oncol* 1997; 15:2238-2246.

55. Leiper AD, Stanhope R, Lau T, Grant DB, Blacklock H, Chessells JM, Plowman PN. The effect of total body irradiation and bone marrow transplantation during childhood and adolescence on growth and endocrine function. *British J Haematol* 1987;67:419-26.

56. Afify A, Shaw PJ, Clavano-Harding A, Cowell CT. Growth and endocrine function in children with acute myeloid leukemia after bone marrow transplantation using busulphan/cyclophosphamide. *Bone Marrow Transpl* 2000;25:253-6.

57. Sanders JE, Pritchard S, Mahoney P, Amos D, Buckner CD, Witherspoon RP, et al. *Blood* 1986; 68: 1129-1135.

58. Le Roith D, Bondy C, Yakar S, Liu JL, Butler A. The somatomedin hypothesis: 2001. *Endocr Rev* 2001;22:53-74.

59. Adan L, Lanversin ML, Thalassinos C, Souberbielle JC, Fischer A, Brauner R. Growth after bone marrow transplantation in young children conditioned with chemotherapy alone. *Bone Marrow Transplant* 1997; 19:253-256.

60. Katsanis E, Shapiro RS, Robison LL, Haake RJ, Kim T, Pescovitz O, Ramsay NKC. Thyroid dysfunction following bone marrow transplantation: Long-term follow-up of 80 pediatric patients. *Bone Marrow Transplant* 1990;5:335-340.

61. Boulad F, Bromley M, Black P, Heller G, Sarafoglou K, Gillio A, *et al*. Thyroid dysfunction following bone marrow transplantation using hyperfractioned radiation. *Bone Marrow Transplant* 1995;15:71-76.

62. Thomas BC, Stanhope R, Plowman PN, Leiper AD. Endocrine function following single fraction and fractionated total body irradiation for bone marrow transplantation in childhood. *Acta Endocrinologica* 1993;128:508-512.

63. Borgstrom B & Bolme P. Thyroid function in children after allogeneic bone marrow transplantation. *Bone Marrow Transplant* 1994;13:59-64.

64. Al-Fair FZ, Colwill R, Lipton JH, Fyles G, Spaner D, Messner H. Abnormal thyroid stimulating hormone (TSH) levels in adults following allogeneic bone marrow transplants. Bone Marrow Transplantation 1997;19:1019-1022.

65. Sklar C, Whitton J, Mertens A, Stovall M, Green D, Marina N, Greffe B, Wolden S, Robinson L. Abnormalities of the thyroid in survivors of Hodgkin's Disease; data from the Childhood Cancer Survivor Study. *J Clin Endocrinol Metab* 2000;85:3227-3232.

66. Kami M, Tanaka Y, Chiba S, Matsumura T, Machida U, Kanda Y, *et al.* Thyroid function after bone marrow transplantation: possible association between immune-mediated thyreotoxicosis and hypothyroidism. *Transplantation* 2001;71:406-11.

67. Benker G, Schäfer U, Herrmanns U, Mahmoud MK, Olbricht T, Schulte HM *et al.* Allogenic bone marrow transplantation in adults: endocrine sequelae after 1-6 years. *Acta Endocrinol*. 1989;120:37-42.

68. Cohen A, Rovelli A, Bakker B, Uderzo C, van Lint M-T, Esperou H, Gaiero A, Leiper AD, Dopfer R, Cahn JY, Merlo F, Kolb HJ, Sociè G. Final height of patients who underwent Bone Marrow Transplantation for

Hematological Disorders during Childhood: a study by the Working Party for Late Effects – EBMT. *Blood* 1999;93:4109-4115.

69. Boulad F, Bromley M, Black P, Heller G, Sarafoglou K, Gillio A, Papadopoulos E, Sklar C. Thyroid dysfunction following bone marrow transplantation using hyperfractionated radiation. Bone Marrow Transplantation 1995;15:71-76.

70. Schulte C, Reinhardt W, Beelen D, Mann K, Schaefer U. Low T3-syndrome and nutritional status as prognostic factors in patients undergoing bone marrow transplantation. *Bone Marrow Transplant* 1998; 22:1171-1178.

71. Chopra IJ, Williams DF, Orgiazzi J, Solomon DH. Opposite effects of corticosteroids on serum concentration of 3,3',5 triiodothyronine (reverse T3) and 3,3',5 triiodothyronine (T3). *J Clin Endocrinol Metab* 1975;41:911-20.

72. Chopra IJ, Huang TS, Beredo A, Solomon DH, Chua Teco GN, Mead JF. Evidence for an inhibitor of extrathyroidal conversion of thyroxin to 3,5,3' triiodothyronine in sera of patients with nonthyroidal illness. *J Clin Endocrinol Metab* 1985;60:666-72.

73. Curtis RE, Rowling RA, Deeg HJ, Shriner DA, Sociè G, Travis LB, *et al.* Solid cancers after bone marrow transplantation. *N Engl J Med* 1997;336:897-904.

74. Sociè G, Curtis RE, Deeg HJ, Sobocinski A, Filipovich AH, Teavis LB*, et al*. New malignant diseases after allogeneic marrow transplantation for childhood acute leukaemia. *J Clin Oncol* 2000; 18:348-357.

75. Pacini F, Voronstova T, Molinaro E, Shovrova E, Agate L, Kuchinskaya E, Elisi R, Demidchik EP, Pinchera A. Thyroid consequences of the Chernobyl nuclear accident. Acta Paediatr 1999; 88(Suppl):23-27.

76. Kyriacou C, Kottaridis PD, Eliahoo J, McKeag N, Bomford J, McGarrigle HH, Linch DC, Mackinnon S, Chatterjee R. Germ cell damage and Leydig cell insufficiency in recipients of nonmyeloablative transplantation for haematological malignancies. *Bone Marrow Transplant* 2003;31:45-50.

77. Legault L, Bonny Y. Endocrine complications of bone marrow transplantation in children. *Pediatr Transplant* 1999;3:60-66.

78. Glucksberg H, Storb R, Fefer A, Buckner CD, Nieman PE, Clift RA, *et al.* Clinical manifestation of graft versus host disease in human recipients of marrow from HLA-matched siblings donors. *Transplantation* 1974;18:295-304.

79. Shulman H, Sullivan KM, Weiden PL, McDonald GB, Strikeer GE, Sale GE, *et al.* Chronic graft-versus-host disease in man: a clinicopathologic study of 20 long term Seattle patients. *Am J Med* 1980;69:204-17.

80. Bostrom L, Ringden O, Jacobson N, Zwaan F, Nilsson B. European multicenter study of chronic graft-versus-host disease. *Transplantation* 1990;49:1100-5.

81. Kier P, Penner E, Bakos S, Kahls P, Lechhner K, Volc-Platzer B, et al. Autoantibodies in chronic GVHD: high prevalence of antinuclear antibodies. *Bone Marrow Transplant* 1990;6:93-6.

82. Siegert W, Stemerowicz R, Hopf U. Antimitochondrial antibodies in patients with chronic graft-versus-host disease. *Bone Marrow Transplant* 1992;10:221-7.

83. Meirow D, Ben Yehuda D, Prus D, et al. Ovarian tissue banking in patients with Hodgkin's disease: is it safe? *Fertil Steril* 1998;69:996-998.

Endocrine disorders during the first year after autologous stem cell transplant

Introduction

Autologous hemopoietic stem cell transplant is performed more frequently than any allogeneic type of organ transplant world-wide. The endocrine system is one of the most common targets of post-transplant complications (1,2). The relative risk of developing endocrine disorders was found to be related to underlying diseases, previous treatments, use of radiation therapies and type of irradiation schedule, post-transplant treatments and age (3,4). It may be difficult for physicians operating outside specialized centers to decide on the timing of investigating endocrine disorders. Moreover, it is also essential to identify the type of disorders that need treatment, as opposed to those that can only be followed up.

Underlying diseases leading to autologous stem cell transplant are mostly lymphoma, leukemia and multiple myeloma. The transplant is preceded by conditioning regimens that consist of high dose antiblastic treatments, with or without total body irradiation, designed to eradicate the underlying hematological disease.

Most of the data currently available on post-transplant endocrine disorders concern the pediatric setting, whereas studies in transplanted adults are still scant. Moreover, in previous studies (5,6) data from patients submitted to allogeneic or autologous transplant were frequently pooled together, with most patients having undergone radiotherapy as a part of conditioning regimens (6-8). Currently, no prospective data exist on endocrine dysfunction in adult survivors from autologous stem cell transplant. Subsequently to our finding of a high prevalence of persistent endocrine dysfunctions in allogeneic setting (9), in 1999 we began endocrine screening in patients undergoing autografting.

The aim of this study was to assess the function in multiple areas of the endocrine system in adult patients with functioning marrow autograft, in disease remission lasting at least one year after transplant, in the attempt to clarify the management of post-transplant endocrine disorders.

Patients and Methods

Patients

Between 1999 and 2002, 110 adult patients received autologous stem cell transplant in our institution. Primary diseases included Hodgkin's disease and non-Hodgkin's lymphoma, acute or chronic myeloid leukemia, chronic lymphocytic leukemia and multiple myeloma. The data relative to 15 patients who had relapsed or died during the first 12 months were excluded; the study sample consisted of the remaining 95 patients. The clinical features of these patients are summarized in **Table 1.**

Table 1. Clinical characteristics of the 95 patients evaluated for endocrine dysfunction after autologous stem cell transplant

Characteristic	Number (%) or Median (range)
Age at stem cell transplant (years)	39 (17-55)
Women/men	48 (51%)/47 (49%)
Disease:	
Hodgkin disease	29 (31%)
acute myeloid leukemia	27 (28%)
non Hodgkin lymphoma	25 (26%)
multiple myeloma	10 (11%)
chronic lymphocytic leukemia	4 (4%)
Previous radiotherapy	26 (27%)
upper/lower half body	16 (17%)/10(11%)
Conditioning regimen:	
Busulphan (16 mg/kg in 4 days) and cyclophosphamide (120 mg/kg in 2 days)	29 (31%)
Carmustine (300 mg/m^2 in 1 day), etoposide (200 mg/m^2 in 4 days), cytarabine (400 mg/m^2 in 4 days), melphalan (140 mg/m^2 in 1 day)	56 (59%)
High dose melphalan (200 mg/ m^2 in 1 day)	10 (11%)
Corticosteroid treatment:	
duration (days)	128.5 (30-730)
cumulative dose (g/m^2)*	6.5 (0.8-13)

*: cumulative dose of corticosteroids is expressed as equivalents of prednisone.

Informed consent was obtained by all subjects and the study was designed in accordance to the Helsinki II Declaration on human experimentation.

The source of the stem cells was an unmanipulated marrow (n=50) or mobilized peripheral blood progenitors (n=45). Mobilization was achieved by chemotherapy and a granulocyte colony-stimulating factor at a dose of 16 μg/kg daily by subcutaneous injection. No patient underwent radiotherapy as part of the conditioning regimen. The conditioning regimen for myeloid leukemia consisted of busulphan and cyclophosphamide administration. The combination of carmustine, etoposide, cytarabine and melphalan was used in patients with lymphomas and chronic lymphocytic leukemia. Patients with myeloma received only a single high dose of melphalan. Twenty-six patients (16 women/10 men) received radiotherapy as a part of lymphoma and myeloma treatment, 16 of them over the neck/thorax, the other 10 over the abdomen/pelvis.

Study protocol

Patients were evaluated 3±0.4 and 12±0.9 months after autografting for all endocrine functions except for the adrenocortical one, which was investigated during the peritransplant period, 7-20 days after corticosteroid withdrawal and monitored as appropriate in patients with adrenal insufficiency. Thyroid function was evaluated again after 6 months, due to the high prevalence of abnormalities found in the 3rd month. Blood samples were obtained between 08:00 and 10:30 a.m. Circulating free thyroxine, free triiodothyronine, thyroid-stimulation hormone (TSH) and autoantibodies toward thyroid, follicular stimulating hormone (FSH), luteinizing hormone (LH), prolactin, adrenocorticotropin hormone, 17-β-estradiol, testosterone, cortisol, dehydro-epiandrosterone sulphate (DHEAS) and insulin-like growth factor I (IGF-I) were measured in a single sample, whereas prolactin was measured as a 2 hr profile (6 samples taken every 20 min). All measurements were performed by commercially available kits: cortisol, testosterone, estradiol and DHEA-S using Immulite, solid phase chemoluminescent enzyme immunoassay (DPC, Los Angeles, CA, USA); FSH and LH with Biodata S,p.A. kit (Rimini, Italy). Serum TSH was measured using an immunoradiometric assay (Delfia, Wallac, Inc. Finland) and free thyroid hormones were determined by radioimmunoassay Lisophase kits (Technogenetics, Milan, Italia). Antithyreoglobulin antibodies were measured with an Ares Serono kit (Milan, Italy) and antiperoxidase antibodies by a DiaSorin kit (Saluggia, Italy). The intra-assay variation coefficient was <3.6% and the inter-assay one <7.8% for all the measurements.

An ultrasound scan of the thyroid gland was performed in all patients using a 7.5 MHz linear transducer. Transparietal pelvic ultrasound was performed in all women, using a 3.5 MHz linear transducer. Spermiogram was performed in 35 men 12 months after autografting; the remaining 12 men refused seminal fluid analysis.

Statistical Analysis

Statistical analysis was performed by the SPSS Inc. (Chicago, Ill.) package. Due to a skewed distribution of most of the data, median and range were used through the text and in the tables. The Mann-Whitney test was used to compare the differences at 3 and 12 months. Age, sex, diagnosis and treatments received were investigated as possible risk factors for endocrine disorders by χ^2 test and by linear or logistic regression analysis, as appropriate. Two-sided *P* values <0.05 were regarded as significant.

Results

Early abnormalities (at 3 months)

Serum IGF-I levels were lower than age-reference values in 53 (56%) of patients (**Table 2**).

Among the 48 women, 42 were in reproductive age: all had had a normal age at menarche, followed by regular periods. Two women underwent bilateral ovariectomy because of ovarian lymphoma or leukemia infiltration; 37 of the remaining 40 women (93%) had secondary hypergonadotropic amenorrhea. Cycles disappeared 3-12 months before autografting in 12 women (32%), consequently to previous antiblastic treatments, and in 25 women (68%) after the conditioning regimens. Only three women (7%) reported normal cycles before and after autografting.

Among the 47 men, one was excluded due to hypogonadotropic hypogonadism of unknown onset. High FSH values were found in 39 out of 46 men (85%) and low testosterone levels in 17 of them (37%). LH levels were normal in all men. Testosterone (p=0.05) and LH (p<0.05) levels were lower in patients with lymphoma than in those with leukemia or

myeloma, likely due to a greater inhibition of the reproductive axis by a prior greater exposure to corticosteroids.

Table 2. Endocrine evaluation in the 95 patients at 3 and 12 months after autologous stem cell transplant

Variable	3 month	12 month	Normal range
	Median (range) or number (%)		
IGF-I	120 (67 – 430)	170 (143 –540)*	100-625 ng/ml†
Women‡	40 (83%)	40 (83%)	
FSH	65 (5.8 – 111)	49.5 (4.9 – 103)	2-13 U/L
LH	28 (2.4 – 77)	17 (3.2 – 60)	2-15 U/L
Prolactin	11 (7 – 16.2)	12.5 (8 – 17.3)	5-20 ng/ml
17β-estradiol	15 (8 - 68)	29.2 (8 – 78)*	40-250 pg/ml
Men	46 (98%)	46 (98%)	
FSH	22 (2.1 – 59)	16.4 (2.1 – 22) *	2-11 U/L
LH	7.5 (2.3 – 40)	6.2 (2.2 – 12)	2-10 U/L
Prolactin	6.9 (5 – 29)	7 (4.5 – 23)	5-20 ng/ml
Testosterone	3.0 (1.8 – 7.9)	4.8 (2.0 – 8.1)*	3.0-10.0 pg/ml
TSH	1.56 (0.25 – 7)	1.6 (0.8 -10.5)	0.3-3.5 mUI/ml;
Triiodothyronine	3.2 (1.9 – 4.4)	3.7 (2.9 – 5.2)*	2.8-5.6 pg/ml
Thyroxine	10. 5 (7 – 15.4)	10.8 (7.3 – 14.8)	6.6-18.0 pg/ml
Subclinical hyperthyroidism	15 (16%)	0*	-
Subclinical hypothyroidism	9 (9%)	11 (12%)	-
"Low T_3" syndrome	29 (30.6%)	0 *	-
Patients with antibodies to thyroid	11 (12%)	16 (16.8%)	-
Number of subjects with antibodies antiperoxidase / antithyreoglobulin / both	5 /5/1	7 /6/3	<10 U/ml and <100 U/ml
Ultrasonographic evidence of chronic thyroiditis	4 (4.2%)	3 (3.15%)	-

† : the normal IGF-I range in men was 180-625 µg/l for <20 yrs of age, 118-475 µg/l for ages 21-30 yrs, 102-400 µg/l for 31-40 yrs, 100-306 µg/l for 41-50 yrs and 95-270 µg/l for 51-60 yrs. In women <20 yrs of age it was 151-530 µg/l, 21-30 yrs: 118-450 µg/l, 31-40 yrs: 100-390 µg/l, 41-50 yrs: 96-288 µg/l and 51-60 yrs: 90-250 µg/l. ‡: only women in reproductive age with ovaries were considered; *: $p<0.01$ *vs.* values at 3 months.

Prolactin was within the normal range in all patients.

Secondary adrenal insufficiency was diagnosed in 28 out of 95 (30%) patients; all of them had been treated with corticosteroids as a part of previous treatment protocols, at a median cumulative dose of 7 g/m^2 for an average period of 130 days. However, a cumulative dose of corticosteroids (7±5 *vs*. 6±4 g/m^2, p=0.3) and the duration of the treatment (126±130 *vs*. 141±200 days; p=0.7) were similar in patients who had or had not developed adrenal insufficiency, suggesting some individual variability in the adrenal axis suppression.

Pre-transplant thyroid function was normal in the 40 patients tested. After the transplant, the "low T_3 syndrome" (thyroxin and TSH within, and triiodothyronine below the normal range) was found in 29 patients (31%). Sub-clinical hyperthyroidism (normal triiodothyronine and thyroxine with TSH below the normal range) was diagnosed in 15 patients(16%). Subclinical hypothyroidism (normal thyroid hormones and TSH 4-7 μU/ml) was diagnosed in nine patients (10%): in one it was transient, while in eight it progressively worsened. Pre-existing thyroid nodules had been reported in 10 patients (11%), while in another 5 cases (5%) the nodules were detected after the transplant; their diameter ranged between 0.7-2 cm (median, 1 cm).

Late abnormalities (at 12 months)

Serum IGF-I levels normalized in 17 out of 53 (18%) patients and remained low in 36 cases (38% of 95).

Four women (11% of 37) experienced a spontaneous recovery of the ovarian function. Estradiol was significantly higher in patients aged <35 yrs than in the older ones (41±19 vs. 12±35 pg/ml; p=0.003) and lower in women conditioned by busulphan/cyclophosphamide when compared to other conditioning regimens (15±5 *vs.* 35±22 pg/ml; p<0.0001). No predictors of recovery for menstrual cycles were found within the first year after autografting. Among the 33 women with persistent amenorrhea, 10 (30%) had a further increase in gonadotropin levels (by 30-100% of baseline) and had no ultrasonographic evidence of ovarian follicles. In 13 other women (39%) ovarian follicles were detected, FSH levels reduced and estradiol increased.

Testosterone levels normalized in 15 men (88%) and remained low only in two. FSH remained above the normal range in 38 (83%) of men. Patients previously treated by pelvic or abdominal radiotherapy had significantly higher FSH levels (22±8 vs. 14 ±4 UI/L; p<0.0001). There were no other predictors of gonadal axis function improvement in men. Abnormalities of seminal fluid analysis were found in all 35 patients: 32 had azoospermia (91%) and three had oligospermia with reduced motility (9%).

Adrenal insufficiency recovered after 3 to 7 months in all patients. Interestingly, cortisol and DHEAS values were still significantly lower in 10 patients previously treated by abdominal/pelvic irradiation, compared to those who had never received radiotherapy (cortisol: 117±54 *vs.* 162±47 ng/ml, p=0.002; normal range, 50-250 ng/ml; DHEAS: 98±59 *vs.* 210±98 mcg/dl, p<0.001; normal range, 80-560 mcg/dl).

The "low T_3 syndrome" disappeared invariably, and triiodothyronine levels normalized in all subjects. Sub-clinical hyperthyroidism diagnosed in the 3rd month was also transient, and had disappeared by the 6-month evaluation. A mild increase in thyroid autoantibodies which is a biochemical evidence of chronic autoimmune thyroiditis, was found in 11 (12%) patients at 3 months and in 16 (17%) at 12 months, all of them had normal thyroid function. The ultrasound showed a non-homogeneous hypoechoic pattern typical of

thyroiditis in only four of them. Three new cases of subclinical hypothyroidism were detected, accounting for a total of 11 (12%) hypothyroid patients. As expected, the frequency of hypothyroidism was higher in patients previously (15-36 months earlier) treated by neck/thoracic radiotherapy than in untreated ones (50% *vs*. 1.3%; $p<0.0001$). Pre-existing nodules disappeared after transplant in two patients and remained stable in all the others, in terms of size and consistency.

Discussion

This prospective study aimed at investigating endocrine dysfunctions during the first year after autologous stem cell transplant; it shows an equal distribution of endocrine disorders among men and women, with multiple glands involved in most cases. Persistent gonadal insufficiency was the most frequent abnormality (>90%). Persistent IGF-I deficiency (38%) and hypothyroidism (12%) were observed less frequently, while peri-transplant hypocortisolism (29%) was transient. A significant prevalence of transient hyperthyroidism (16%) was also documented. Since we do not have data on endocrine function before transplant for all patients, except for adrenal function and menstrual disorders, we cannot exclude that some dysfunctions preceded the transplant procedure. Since radiotherapy was not used in conditioning regimens and only few patients had previously received irradiation, we are unable to speculate as to whether endocrine system damage may have been due to different doses or regimens of radiotherapy.

While large studies on growth hormone-IGF-I axis disorders have been reported in children (12-14), scarce data are available in adults. In this study we did not perform any stimulation test to evaluate the growth hormone secretory potential, therefore, we cannot discuss this issue. We measured, however, circulating IGF-I levels and we found them significantly decreased in 56% of patients 3 months after the transplant, with a recovery in 18% of them, within 12 months. The recovery can likely be explained by the loss of the inhibitory effect of previous glucocorticoid treatment on the somatotropic axis, and by an improved metabolic state late after autografting (15).

Gonadal damage affected virtually all patients, with a few exceptions. The dominant manifestation was hypergonadotropic amenorrhea in women and spermatogenesis injury in

men. Both alkylating agents and irradiation cause germ cell injury and damage to Leydig cells in men, and to the follicular pattern in women (3,18). Indeed, women conditioned by busulphan/cyclophosphamide had lower estradiol levels than those conditioned by the other regimens. Menstrual cycles recovered in only 7 (18%) out of 40 women within the 12th month, while later recovery may be possible in some of the other 13 women with ovarian follicles at pelvic ultrasonography (10,20). Younger age has often been associated with higher likelihood of reproductive function recovery (9,10,21); this finding did not emerge clearly in our cohort up to 12 months after transplant. However, higher estradiol levels in younger patients indicate better residual ovarian function.

Impaired spermatogenesis was documented in all patients who underwent seminal fluid analysis at 12 months, 91% of them showed germinal aplasia with azoospermia. Increased FSH levels were found in 85% of men, indicating that spermatogenesis damage was not always associated with FSH increase. Increased FSH levels up to 5 years were previously found in 2/3 of auto-transplanted men (22), and spermatogenesis resumed later than 12 months after allografting (23); thus, some patients will likely have spermatogenesis recovery later on. In our cohort, radiotherapy was associated with significantly higher FSH levels, suggesting a greater testicular injury in patients treated by abdominal/pelvic irradiation. Similarly, a greater cumulative probability for germ cell injury was previously found in patients treated by radiotherapy than in those treated by chemotherapy only (6,24). When concerning with the hormonal pattern, the data is controversial, indicating either persistently low or normal testosterone levels in long-term survivors to autografting (17,25,26).

Overt adrenal insufficiency was principally related to corticosteroid treatment as part of previous cytotoxic regimens for the underlying diseases. No stimulation test was performed in this study; therefore, a milder degree of adrenal insufficiency cannot be excluded in other patients. Although serum cortisol recovered within the normal range after a period of appropriate replacement therapy, previous radiotherapy was associated with lower cortisol and DHEAS levels after 12 months. Only the initial report by Sanders *et al*. (27) described a high incidence of adrenocortical dysfunction following total body irradiation and stem cell transplant; subsequent studies found rare evidence of permanent post-transplant cortisol deficiency (4,28). Other authors have reported a persistent (up to 5 years) decrease in adrenal androgens in children conditioned by radiotherapy, despite their normal cortisol values (29). In the light of these findings, radiotherapy, although not causing permanent adrenal

insufficiency, can cause long-lasting partial injury to the adrenocortical tissue, expressed principally as reduced androgen production.

Thyroid abnormalities were more frequent in our sample (66%) than in those of previous studies (28,30,31) where thyroid dysfunctions were mostly ascribed to radiotherapy. Carlson *et al*. described thyroid disorders in only 18% of patients, 3 to 60 months after autografting (30). In our cohort, subclinical disorders were also considered. Subclinical hyperthyroidism was transient, i.e., limited to the period of immunological reconstitution occurring within 3 months after the transplant. Transient thyreotoxicosis was reported during the same period (peak, 100 days) after allografting, and an immune induced injury was claimed as the major pathogenic factor (31). A similar disorder, although less expressed, may also occur after autografting and the difference in severity can be related to a milder degree of immune system derangement after autologous transplant (32,33). Subclinical hypothyroidism was mostly related to previous radiotherapy and worsened progressively in all but one patient. In analogy with our finding, other authors also described busulphan/cyclophosphamide conditioning to be linked to thyroid dysfunction and TSH increase, with a similar incidence (11%)(34). Biochemical evidence of thyroiditis was found in 17% of patients, while a typical ultrasonographic pattern was observed in only 4%. Further investigation is needed to clarify this discrepancy. Elevated thyroid antibody titers also appeared up to 12 months in 4/111 auto-transplanted patients (30).

Transient "low T_3 syndrome" was likely part of the negative metabolic condition that persisted several months after transplant, and disappeared thereafter. Corticosteroid and antiblastic treatments may also contribute to this thyroid dysfunction (35-39). Thyroid nodules were mostly small and stable over follow-up.

Frequent post-transplant endocrine disorders raise an important clinical question of when and how to treat them. Transiently low IGF-I does not always mean growth hormone deficiency in transplanted patients. The deficiency should be investigated after resolving the post-transplant negative metabolic situation and after an adequate interval from corticosteroid withdrawal. It is still unclear whether replacement treatment may be helpful in growth hormone deficient adults after transplant. Hypergonadotropic amenorrhea often seems irreversible, and should be treated by hormone replacement or oral contraceptive therapies in all reproductive aged women, unless contraindicated. As spontaneous pregnancies have been

reported in auto-transplanted women on hormonal replacement therapy (40), these patients should be advised to use other contraceptive measures, especially in the presence of ovarian follicles. Hormone therapy should be withdrawn for a period of 2-3 months a year, monitoring the reproductive axis function. It is still unclear to which extent mild transient male hypogonadism may influence general health condition and whether replacement treatment may be helpful. Generally, it is left untreated, although some reports indicate a relationship between transient low testosterone levels and bone mass decrease (41,42). Spermatogenesis hardly recovers within 12 months after autografting and should be monitored every 6-12 months thereafter. Adrenal insufficiency is still an underestimated condition; albeit transient, it can impair post-transplant recovery. Replacement therapy with short-acting steroids is mandatory, with a progressive reduction of the dose, in order to enable the adrenal axis to gradually recover. When concerning with thyroid function, we recommend to perform the first evaluation within 3 months after autografting. Patients with a completely normal picture should undergo annual assessment of thyroid hormones, thyroid specific autoantibodies and ultrasonography. Transient hyperthyroidism is usually non symptomatic in auto-transplanted patients, and does not require any treatment; the same is true for the "low T_3 syndrome." Nevertheless, these patients should be monitored every three months until their endocrine values are normalized. Patients with thyroiditis and normal thyroid function should be monitored every 3-4 months, due to their risk in developing hypothyroidism. Hypothyroidism needs replacement treatment, since TSH increase is considered as the main regulator of thyreocyte differentiation and proliferation (43). While it is well established that external irradiation of the neck may predispose to hypothyroidism and thyroid neoplasms, the natural history, timing and peak incidence in adults are still unknown (3,44-46). This issue is even less clear for patients who have undergone chemotherapy and transplant procedures. Therefore, all patients should undergo life-long yearly evaluations of thyroid function and morphology.

In conclusion, this study documents frequent endocrine disorders during the first year after autologous stem cell transplant. Despite the tendency to improve, in more than half of the cases, the complications persist over one year. Multiple factors including previous antiblastic, corticosteroid and radiation treatments, an abnormal immunological condition and general health status are likely responsible for the onset of endocrine disorders. Precocious gonadal failure, adrenal insufficiency, thyroiditis and hypothyroidism require specific

monitoring and treatment by specialized operators. Therefore, endocrine screening is necessary during the first year after autologous stem cell transplant.

References

1. Kolb HJ, Socié G, Duell T, et al. For the late effects working party of the European cooperative group for blood and marrow transplantation and the European late effects project group. Malignant neoplasms in long-term survivors of bone marrow transplantation. *Ann Intern Med.* 1999;131:738-744.
2. Cohen A. Late complications of transplants. Endocrinological complications. In: Blood and Marrow Transplantation 1998 (The European School of Haematology Handbook 1998) Eds: J Apperley, E. Gluckman, A. Gratwohl Associate Editor: C. Craddock. Paris, pp 154-159.
3. Shalet SM, Didi M, Oglivy-Stuart AL, Schulga J, Donaldson MDC. Growth and endocrine function after bone marrow transplantation.[Review] *Clin Endocrinol.* 1995;42:333-339.
4. Brennan BMD, Shalet SM. Endocrine late effects after bone marrow transplant. [Review] *Br J Haematol.* 2002;118:58-66.
5. Grigg AP, McLachlan R, Zaja J, Szer J. Reproductive status in long-term bone marrow transplant survivors receiving busulphan-cyclophosphamide (120 mg/kg). *Bone Marrow Transplant*. 2000;26:1089-1095.
6. Mertens AC, Ramsy NKC, Kouris S, Neglia JP. Patterns of gonadal dysfunction following bone marrow transplantation. *Bone Marrow Transplant*. 1998;22:345-350.
7. Boulad F, Bromley M, Black P, et al. Thyroid dysfunction following bone marrow transplantation using hyperfractionated radiation. *Bone Marrow Transplant.* 1995;15:71-76.
8. Giorgiani G, Bozzola M, Locatelli F, et al. Role of busulphan and total body irradiation on growth of prepubertal children receiving bone marrow transplantation and results of treatment with recombinant human growth hormone. *Blood*. 1995;86:825-831.
9. Tauchmanovà L, Selleri C, De Rosa G, et al. High prevalence of endocrine dysfunction in long-term survivors after allogeneic bone marrow transplantation for hematological diseases. *Cancer.* 2002; 95:1076-1084.
10. Tauchmanovà L, Selleri C, De Rosa G, et al. Gonadal status in reproductive age women after allogeneic or autologous stem cell transplantation. *Hum Reprod*. 2003, 18:1410-1416.
11. Trendelenburg M, Gregor M, Passweg J, Tichelli A, Tyndall A, Gratwohl A. Altered immunity syndrome, a distinct entity in long-term bone marrow transplantation survivors? *Bone Marrow Transplant*. 2001;28:1175-1177.
12. Afify A, Shaw PJ, Clavano-Harding A, Cowell CT. Growth and endocrine function in children with acute myeloid leukemia after bone marrow transplantation using busulphan / cyclophosphamide. *Bone Marrow Transplant.* 2000;25:253-6.
13. Leiper AD, Stanhope R, Lau T, et al. The effect of total body irradiation and bone marrow transplantation during childhood and adolescence on growth and endocrine function. *British J Haematol.* 1987;67:419-26.
14. Cohen A, Rovelli A, Bakker B, et al. Final height of patients who underwent Bone Marrow Transplantation for Hematological Disorders during Childhood: a study by the Working Party for Late Effects – EBMT. *Blood.* 1999;93:4109-4115.
15. Le Roith D, Bondy C, Yakar S, Liu JL, Butler A. The somatomedin hypothesis: 2001. *Endocr Rev*. 2001;22:53-74.
16. Adan L, Lanversin ML, Thalassinos C, Souberbielle JC, Fischer A, Brauner R. Growth after bone marrow transplantation in young children conditioned with chemotherapy alone. *Bone Marrow Transplant.* 1997;19:253-256.

17. Littley MD, Shalet SM, Morgenstern GR, Deakin DP. Endocrine and reproductive dysfunction following fractionated total body irradiation in adults. *Quarterly J Medicine*. 1991;287:265-274.

18. Shalet SM. 1994 Cancer therapy and gonadal dysfunction. In: Clinical Endocrine Oncology (ed. by R. Sheaves, P. Jenkins & JAH Wass) pp 510-513. Blackwell Science Ltd, Oxford, UK.

19. Chatterjee R, Mills W, Katz M, McGarrigle HH, Goldstone AH. Germ cell failure and Leydig cell insufficiency in post-pubertal males after autologous bone marrow transplantation with BEAM for lymphoma. *Bone Marrow Transplant*. 1994;3:519-522.

20. Herschlag A, Schuster M. Return of fertility after autologous stem cell transplantation. *Fertil Steril.* 2002; 77:419-421.

21. Schimmer AD, Quatermain M, Imrie K, et al. Ovarian function after autologous bone marrow transplantation. *J Clin Oncology.* 1998;16:2359-63.

22. Keilholtz U, Max R, Scheibenbogen C, Wüster Ch, Körbling M, Haas R. Endocrine function and bone metabolism 5 years after autologous bone marrow/blood-derived progenitor cell transplantation. *Cancer.* 1997;79:1617-22.

23. Anserini P, Chiodi S, Spinelli S, Costa M, Conte N, Copello F, Bacigalupo A. Semen analysis following allogeneic bone marrow transplantation. Additional data for evidence-based counselling. *Bone Marrow Transplant*. 2002;30:447-451.

24. Chatterjee R, Kottaridis PD, McGarrigle HH, Papatryphonos A, Goldstone AH. Hypogonadism fails to prevent severe testicular damage induced by total body irradiation in a patient with beta-thalassaemia major and acute lymphoblastic leukemia. *Bone Marrow Transplant*. 2001;28:989-991.

25. Schimmer AD, Ali V, Stewart AK, Imrie K, Keating A. Male sexual function after autologous blood or marrow transplantation. *Biol Blood Marrow Transplant*. 2001;7:279-83.

26. Molassiotis A, van den Akker OBA, Milligan DW, Boughton BJ. Gonadal function and psychosexual adjustment in male long-term survivors of bone marrow transplantation. *Bone Marrow Transplant*. 1995;16:253-259.

27. Sanders JE, Pritchard S, Mahoney P, et al. Growth and development following marrow transplantation for leukemia. *Blood*. 1986; 68:1129-1135.

28. Ogilvy-Stuart AL, Clark DJ, Wallace WHB, et al. Endocrine deficit after fractionated total body irradiation. *Arch Dis Child.* 1992;67:1007-1010.

29. Bolme P, Borgstrom B, Carlstrom K. Longitudinal study of adrenocortical function following allogeneic bone marrow transplantation in children. *Horm Res*. 1995;43:279-285.

30. Carlson K, Lonnerholm G, Smedmyr B, Oberg G, Simonsson B. Thyroid function after autologous bone marrow transplant. *Bone Marrow Transplant*. 1992;10:123-127.

31. Kami M, Tanaka Y, Chiba S, et al. Thyroid function after bone marrow transplantation: possible association between immune-mediated thyreotoxicosis and hypothyroidism. *Transplantation*. 2001;71:406-11.

32. Sklar C, Boulad F, Small T, Kernan N. Endocrine complications of pediatric stem cell transplantation. *Front Biosci*. 2001;1:6:G17-22.

33. Tauchmanovà L, Selleri C, Rotoli B, Lombardi G, Colao A. Endocrine consequences of stem cell transplantation for hematological malignancies. In: Research Advances of Cancer 3. 2003, pp207-217.

34. Al-Fair FZ, Colwill R, Lipton JH, Fyles G, Spaner D, Messner H. Abnormal thyroid stimulating hormone (TSH) levels in adults following allogeneic bone marrow transplants. *Bone Marrow Transplant*. 1997;19:1019-1022.

35. Toubert ME, Sociè G, Gluckman E, et al. Short- and long-term follow-up of thyroid dysfunction after allogeneic bone marrow transplantation without the use of preparative total body irradiation. *Br J Haematol*. 1997;98:453-57.

36. Reinhardt W, Sauter V, Jockenhovel F, et al. Unique alteration of thyroid function parameters after i.v. administration of alkylating drugs (cyclophosphamide and ifosfamide). *Exp Clin Endocrinol Diabetes*. 1999;107:177-82.

37. Chopra IJ, Williams DF, Orgiazzi J, Solomon DH. Opposite effects of corticosteroids on serum concentration of 3,3',5 triiodothyronine (reverse T3) and 3,3',5 triiodothyronine (T3). *J Clin Endocrinol Metab*. 1975;41:911-20.

38. Chopra IJ, Huang TS, Beredo A, et al. Evidence for an inhibitor of extrathyroidal conversion of thyroxin to 3,5,3' triiodothyronine in sera of patients with nonthyroidal illness. *J Clin Endocrinol Metab.* 1985;60:666-72.

39. Schulte C, Reinhardt W, Beelen D, Mann K, Schaefer U. Low T3-syndrome and nutritional status as prognostic factors in patients undergoing bone marrow transplantation. *Bone Marrow Transplant*. 1998; 22:1171-1178.

40. Hershlag A, Schuster M. Return of fertility after autologous stem cell transplantation. *Fertil Steril.* 2002;77:419-421.

41. Gandhi MK, Lekamwasam S, Inman I, et al. Significant and persistent loss of bone mineral density in the femoral neck after haematopoietic stem cell transplantation: long-term follow-up of a prospective study. *Br J Haematol*. 2003;121:462-468.

42. Kananen K, Volin L, Tähtelä R, et al. Recovery of bone mass and normalization of bone turnover in long-term survivors of allogeneic bone marrow transplantation. *Bone Marrow Transplant.* 2002;29:33-39.

43. Rivas M, Santisteban P. TSH-activated signaling pathways in thyroid tumorigenesis. *Mol Cell Endocrinol.* 2003;213:31-45.

44. Curtis RE, Rowling RA, Deeg HJ, et al. Solid cancers after bone marrow transplantation. *N Engl J Med*. 1997;336:897-904.

45. Pacini F, Voronstova T, Molinaro E, et al. Thyroid consequences of the Chernobyl nuclear accident. *Acta Paediatr.* 1999(Suppl);88:23-27.

46. Baker SK, Gurney JG, Ness KK, et al. Late effects in survivors of chronic myeloid leukemia treated with hematopoietic cell transplantation: results from the Bone Marrow Transplant Survivor Study. *Blood*. 2004;104:1898-1906.

High prevalence of endocrine dysfunction in long-term survivors after allogeneic bone marrow transplantation for hematological diseases

Introduction

Bone marrow transplantation (BMT) programs started in Europe in the early 1970s as highly experimental procedures and have now progressed to become the treatment of choice for a wide variety of diseases. While in the beginning severe aplastic anemia was the main indication for allogeneic BMT (allo-BMT), currently leukemia, followed by other hematological and non hematological diseases is the most frequent disorder treated by this procedure (1).

As the number of long-term survivors after allo-BMT is progressively increasing, attention is now focused on early and late complications of this procedure which may worsen the quality of life and/or threaten the long-term outcome of transplant recipients (1). A high risk of bone complications (2), thyroid (3-5) and gonadal dysfunction (6,7) has been described in adult survivors. The relative risk of these complications is likely to be influenced by the underlying disease, previous treatments, post-BMT treatments and age at BMT (7). Marrow transplant conditioning regimens are designed to eradicate the underlying hematological disease (mostly a malignancy) and to suppress the host immune system. They consist in highly aggressive antiblastic treatments. Moreover, great amounts of cytokines are released at the time of transplantation (8), and immune deficiency occurs due to delayed and incomplete recovery of the immune system. Graft-versus-host disease (GvHD) causes both stimulation and suppression of the immune system, the latter due to either direct immunosuppressive effects or consequence of the drugs administered to treat the disease (9). Prevention and treatment of GvHD consist in high-dose immunosuppressive treatments including glucocorticoids, methotrexate and cyclosporine A. Consequently, the integrity of several systems and tissues, including the endocrine function may be affected by multiple exogenous and endogenous damaging factors.

Most of the data concerning the endocrine function derive from the evaluation of patients who underwent total body irradiation (TBI) as part of pre-transplantation treatments. At present, the combination of busulphan and cyclophosphamide is largely employed in conditioning regimens. However, only a few studies have been carried out in adults treated without TBI (5,10); consequently, the impact of chemotherapy and immune system derangement on the endocrine function in present allo-BMT recipients is still not completely elucidated.

As transplanted patients may ask for medical attention outside the highly specialized institutions where they were transplanted, cross sectional or prospective studies are essential to evaluate the actual prevalence of non hematological late effects. The main aim of this study was to assess the function in multiple areas of the endocrine system in adult long-term survivors with functioning donor marrow graft transplanted for hematological diseases and conditioned without the use of TBI.

Patients and Methods

Patients

One hundred and six patients received bone marrow-derived stem cells from HLA-identical siblings in our institution since 1994. Only patients surviving more than 1 year after BMT, disease-free and with complete hematological reconstitution at the time of the endocrine evaluation and giving informed consent, were included in the study. Forty consecutive patients (21 F, 19 M) met the inclusion criteria and entered the study. Their age at transplantation ranged between 13 and 45 years (median, 29) and their post-BMT follow-up lasted from 12 to 62 months (median, 38). Primary diseases were acute (n=17) or chronic (n=16) myelocytic leukemia, acute lymphoblastic leukemia (n=5), and severe aplastic anemia (n=2).

Treatments

All patients received the myeloablative conditioning regimen BUCY2 including busulphan (16 mg/kg in 4 days) and cyclophosphamide (120 mg/kg in 2 days). Acute GvHD prophylaxis was performed with cyclosporin A (1 mg/kg i.v. from day –1 to +21, then 10 mg/kg p.o. for 6 months) and “short course” methotrexate (10 mg/kg for 4 doses).

Acute GvHD was treated by high-dose methylprednisolone (2-10 mg/kg for 10 days), followed by slow dose tapering in the following 6 months, monitoring patients' clinical conditions. Chronic GvHD was treated by prednisolone at doses of 1-2 mg/kg, associated with CsA at doses ranging from 1 to 8 mg/kg/day. The median cumulative dose of prednisone equivalents was 10 g/m^2 (range 0.9-24 g/m^2). Previous treatment history included alkylating agents only for patients with ALL, while radiotherapy before transplant was an exclusion criteria (**Table 1**).

Table 1. Clinical characteristics of 40 patients evaluated for endocrine dysfunction after allogeneic bone marrow transplant

Patient characteristics	N=40
Age at transplant (years)	29 (13-45)
Women/men	21/19
Basic disease:	
acute myeloid leukemia (AML)	17
chronic myeloid leukemia CML)	16
acute lymphoblastic leukemia (ALL)	5
severe aplastic anemia (SAA)	2
Previous treatment history:	
AML	IDA/MIT/CYT/ETO
CML	HYD/IFN-□
ALL	VCR/DNR/CYC/CYT/MTX/MIT/ASP
SAA	No therapy
Conditioning regimen:	
BUCY2	38
CY	2
Follow-up duration (months)	38 (12-62)
N° of patients with acute GVHD	13
N° of patients with chronic GVHD	26
limited/extensive form	15/11
Corticosteroid treatment:	
Duration (months)	9 (3-36)
Cumulative dose (g)*	10 (0.9-24)
Cyclosporine A treatment:	
Duration (months)	6.65(3.3-26.3)
Cumulative dose (g)	59.5(36-300)

The values are expressed as median and range. Abbreviations: BU: busuphan, CY: cyclophosphamide, IDA: idarubicin; MIT: mitoxantrone; CYT: cytarabine; ETO: etoposide; VCR: vincristine; DNR: daunorubicin; CYC; cyclophosphamide; MTX: methotrexate; HYD: hydroxyurea; ASP: asparaginase; GVHD: graft *vs* host disease. *: cumulative dose of corticosteroids is expressed as prednisone equivalents.

Acute GvHD was graded according to the Glücksberg system (11), and chronic GvHD was graded as either limited or extensive, based upon clinical severity and target organ involvement (12). Thirteen patients had been affected by acute GvHD, and twenty-six

patients were affected by chronic GvHD (15 limited and 11 extensive form) at time of evaluation. All these patients were treated by corticosteroids for a period ranging from 6 to 36 months. Eleven patients with extensive chronic GvHD were also treated with cyclosporine A at a median cumulative dose of 59.5 gr (range, 36 to 790 gr), in order to reduce the corticosteroid maintenance dose.

Methods

More than 75% of the patients included in the study were transplanted after 1995 and were prospectively evaluated since transplantation. The remaining patients were investigated in a cross-sectional way at first, then they were followed-up prospectively. In order to combine the data of these two groups of patients, the first evaluation of those transplanted before 1995 and the evaluation at 12 months in those transplanted since 1995, were pooled. However, some data obtained in the prospective study were also included, when a more complete information could be obtained on the endocrine function. For the same reason, when some previous medical record on endocrine function was available and gave important insight on patient outcome, this was also considered.

Blood samples were obtained between 08:00 and 10:30 a.m. To assess the thyroid, gonadal, adrenal and somatotroph axes, circulating free thyroxine (FT4), free triiodothyronine (FT3), thyroid stimulation hormone (TSH) and circulating autoantibodies toward thyroid, FSH, LH, ACTH, 17-β-estradiol, testosterone, cortisol, dehydroepiandrosterone sulphate (DHEA-S) and insulin-like growth factor I (IGF-I) were measured in a single sample while prolactin (PRL) and GH were measured during a 2 hr profile (6 samples taken every 20 min). All measurements were performed by commercially available kits: cortisol, testosterone, estradiol and DHEA-S using Immulite, solid phase chemoluminescent enzyme immunoassay (DPC, Los Angeles, CA, USA); FSH and LH with RIA Biodata S,p.A. (Rimini, Italy). ACTH was tested by double-antibody ^{125}I radioimmunoassay (DPC Los Angeles, CA 90045-5597, USA). Serum TSH levels were measured using an immunoradiometric assay (IRMA) (Delfia, Wallac, Inc. Finland) which has a detection limit of 0.03 mU/L. Serum concentrations of FT3 and FT4 were determined by RIA Lisophase kit (Technogenetics, Milan, Italia). Antithyreoglobulin antibodies were measures with an immunoradiometric technique (Ares Serono kit set, Ares Serono, Milan, Italy), while antiperoxidase antibodies were assayed by a RIA kit (DiaSorin, Inc, Saluggia, Italy). Serum GH levels were measured by IRMA using commercially available kits (Sorin, Saluggia, Italy); the sensitivity of the

assay was 0.2 μg/L. IGF-I was measured by IRMA after ethanol extraction; the sensitivity of the assay was 0.8 μg/L. The normal IGF-I range in our laboratory was 141-680, 130-625, 110-450 and 100-300 μg/L for patients pubertal (13-16 yrs), adolescent, and aged 20-40, 41-59 yrs, respectively.

Ultrasound scan of the thyroid gland was performed in all patients using a 7.5 MHz linear transducer. Previous medical records were reviewed to obtain complete information on patients' outcome including data on menstrual history pre- and post-transplantation. At least one measurement was performed for GH/IGF-I, thyroid and adrenal function determination. The gonadal function was investigated at least twice in both genders; in women one baseline measurement and another on HRT treatment were performed, while in men two baseline measurements were performed in order to distinguish a transient from a persistent damage. When multiple samples were available for the same patient, the mean value was calculated. Informed consent was obtained from all patients and the design was made in accordance with the Helsinki II declaration**.**

Statistical Analysis

Because the data had a skewed distribution, median and range were used throughout the text and in tables. The Mann-Whitney test was used to compare the differences between the groups with and without GVHD; the χ^2 test was used to assess association of endocrine disorders with particular clinical features. Two-sided *P* values <0.05 were regarded as significant.

Results

The hypothalamus-pituitary-gonadal axis

Women enrolled in the study had previous history of normal age at menarche followed by regular periods. Following BMT, all but two women had permanent ovarian insufficiency (secondary hypergonadotropic amenorrhea). Two of them had became menopausal several months before allografting due to previous intensive chemotherapy. During amenorrhea, PRL levels were normal, FSH and LH values were high and 17-β-estradiol was often under the detection limit of the assay (**Table 2**).

Table 2. Hypothalamus-pituitary -ovarian axis evaluation in 21 transplanted women

Variable	Baseline	HRT*	Normal range
FSH	120 (50 – 259)	2,65 (0.3 – 30)	2-13 U/L
LH	78 (16 – 151)	4.2 (0.5 – 20)	2-15 U/L
Prolactin	11 (7 – 16.2)	12.5 (8 – 17.3)	5-20 ng/ml
17β-estradiol	< 20	62 (35 – 88)	40-250 pg/ml

Values are expressed as median and range; *: values on cyclic hormone replacement therapy (HRT) with estrogens and progestins were obtained in 20 women.

The youngest woman, 13 yr at BMT, experienced a spontaneous recovery of the ovarian function with regular menstrual cycles after 12 months of amenorrhea. Another woman had transient hypergonadotropic polymenorrhea (FSH = 59.6 UI/l; LH = 32.3 UI/l). In all ameno-rrhoic patients cyclic oral hormone replacement treatment (HRT) was initiated within 12 months after BTM unless controindicated. In the patient with polymenorrhea consequent to BMT, regular cycles were obtained by norethisterone acetate administration for 6 months (10 mg/day for 12 days a month), then permanent amenorrhea occurred and HRT was initiated. Gonadotropin and 17-β-estradiol levels improved significantly in all women on HRT. Fifteen women assumed estradiol (2-3 mg/day) and dihydrogesterone (**Table 2**). Five women, aged >40 yr, were treated by transdermal estradiol administration (50-100 μg delivered/day) associated with oral medroxyprogesterone acetate or nomegestrol (10 and 5 mg/day respectively) 12 days/month. No difference in gonadotropin values was found between women treated by oral or transdermal estradiol administration (data not shown). However, 3 women could not be treated for 20-34 months following allografting because of severe or recurrent chronic liver GVHD, and other 4 women were likely under-treated probably due to reduced intestinal absorption for a mild chronic intestinal GVHD that was confirmed by biopsy in 2 of them. One woman had endometrial damage: no menstrual cycles occurred after cyclic HRT and no endometrium was shown by endovaginal pelvic ultrasound study. One pregnancy was obtained with complete pharmacological assistance and 2 healthy twins were born to a woman 5 years after BMT.

Men had normal LH and testosterone levels, while 9 of 19 (47%) presented FSH values above the upper limit of the normal range (**Table 3**), indicating spermatogenesis damage. By seminal fluid analysis azoospermia was diagnosed in 3 men, one of them having FSH within the normal range. The remaining men refused seminal fluid analysis. No relationship was found between FSH levels increase and age. One pregnancy was

reported in a wife of a man who received allograft 3 years earlier and resulted to have normal FSH levels.

Table 3. Hypothalamus-pituitary-testis axis function in 19 transplanted men

Variable	Baseline	Normal range
FSH	15.1 (3.2 – 20)	2-15 U/L
LH	5.05 (3.5 – 9)	2-10 U/L
Prolactin	10.6 (6 – 15.1)	5-20 ng/ml
Testosterone	5.07 (4 – 11)	3.0-10.0 pg/ml

Values are expressed as median and range.

The hypothalamus-pituitary-adrenal (HPA) axis

Secondary adrenal insufficiency was diagnosed in 4 subjects, all of whom had been treated with corticosteroids for ≥ 10 months for chronic GVHD, at a cumulative dose > 10 gr/m^2. A severe herpes zoster infection occurred in two patients with chronic GVHD causing corticosteroid treatment withdrawal; these patients subsequently showed further worsening of their clinical condition due to acute adrenal crisis. In the four patients with insufficiency, serum cortisol, ACTH levels and urinary cortisol excretion were significantly lower than those of the remaining patients. Adrenal insufficiency recovered after 6 to 18 months in all patients. Cortisol and ACTH levels were within the normal range in 22 patients who were evaluated 30-180 days after corticosteroid treatment withdrawal **(Table 4**). DHEA-S levels were low in the patients with adrenal insufficiency and within the normal range in the remaining patients**.** No significant difference was found in the dose of prednisone equivalents administered or treatment duration between patients developing or not adrenal failure.

Table 4. Hypothalamus-pituitary-adrenal axis evaluation

Variable	Adrenal insufficiency (n=4)*	No insufficiency (n= 36)**	Normal range
Cortisol	5 (2 – 39)	188 (120 – 350)	50–250 ng/ml
ACTH	44.9 (33 – 183)	29 (8 – 58)	9- 50 pg/ml
DHEA-S	57 (39 – 89)	241 (157 – 378)	80 – 560 µg/dl
Cortisol urinary excretion	50 (29 – 50)	105 (60 – 140)	50-150 mcg/24h
Prednisone cumulative dose (g)	15 (11 – 24)	8.9 (1.2 – 15)	-
Corticosteroid treatment duration (mos)	18 (12 – 36)	10 (5 – 15)	-

DHEA-S: dehydroepiandrosterone sulphate; *: the values were obtained 10 to 30 days after corticosteroid withdrawal; **: values were obtained 30 to 180 days after corticosteroid withdrawal.

The hypothalamus-pituitary-growth hormone axis

GH profile showed levels within the normal range in all subjects in study, whereas IGF-I levels were lower in the group of 26 patients affected by chronic GVHD **(Table 5)**. IGF-I levels were lower than age-reference values in 10 (38%) patients who were affected by chronic GVHD and only in one (7%) who was not affected by the complication.

Table 5. Hypothalamus-pituitary-growth hormone axis evaluation

Variable	Patients	Normal range
GH (all patients)	1.2 (0.9 – 2.1)	0.1 – 7 ng/ml
IGF-I (all patients)	154 (50 – 240)	100-685 ng/ml#
IGF-I (GVHD + patients)	94 (50 – 160)*	100-685 ng/ml#
IGF-I (GVHD – patients)	184 (82 – 240)	100-685 ng/ml#

The data are expressed as median and range. GH: growth hormone; IGF-I: insulin-like growth factor –I; **#:** the normal IGF-I range was 141-685, 130-625, 110-450 and 100-300 for patients pubertal (13-16 yrs), adolescents, and aged 20-40 and 41-59 yrs, respectively. ***:** $p<0.05$ *vs* GVHD negative patients.

The hypothalamus-pituitary-thyroid axis

Twenty of 40 patients had had a pre-transplant thyroid function evaluation that resulted normal. After BMT, low T3 syndrome (FT4 and TSH within, and FT3 below their respective ranges of normality) was found in 4 patients with chronic extensive GVHD 12 to 48 months after BMT. Sub-clinical hyperthyroidism (normal FT3 and FT4 levels and TSH values below the normal range) was diagnosed in 6 patients between the 12th and 18th month after BMT. Three of them were re-evaluated after a subsequent period of 4 to 7 months, and TSH normalized. Thyroid ultrasound revealed non-homogeneous hypoechoic pattern in 2 of them. Thyroid autoantibodies were present in only one patient at the diagnosis of sub-clinical hyperthyroidism. Post-transplant thyroid ultrasound showed non-homogeneous hypoechoic pattern in 9 patients, in 6 of them associated with a mild increase in autoantibodies toward thyroid that suggested chronic autoimmune thyroiditis (**Table 6**).

Table 6. Hypothalamus-pituitary-thyroid axis evaluation

Variable	Transplanted patients (n = 40)	Normal range
TSH	1.35 (0.1 – 7)	0.3-3.5 mUI/ml;
FT3	3.05 (1.9 – 5)	2.8-5.6 pg/ml
FT4	11 (6.7 – 13.5)	6.6-18.0 pg/ml
N° of subjects with antibodies antiperoxidase/antithyreoglobulin	5/2	< 10 U/ml/<100 U/ml
N° of patients with antibodies to thyroid	6/40 (15%)	

The data are expressed as median and range. The values are those obtained from the prospective and cross- sectional study; those obtained from medical records are non included in this table.

Sub-clinical hypothyroidism (thyroid hormone within the normal range; TSH 7 μU/ml) was revealed in one of them 5 years after BMT. A similar abnormality was revealed also in another woman with negative autoantibodies (TSH, 5.6 μU/ml) 6 years after BMT. L-thyroxin replacement treatment was given to both patients. Pre-existing thyroid nodules were detected in 2 patients (single nodule in one and 2 small nodules in other). They were stable over time in both patients (follow-up, 18 and 24 months) in terms of size and consistence. One patient developed thyroid lymphoma 8 months after BMT.

Thyroid function, adrenal function and IGF-1 impairments occurred more frequently in patients with than in those without chronic GVHD (**Table 7**).

Table 7. Occurrence of endocrine disorders according to chronic graft-versus-host-disease (GVHD)

Variable	cGVHD + (n = 26)	cGVHD – (n = 14)
Age at transplant (years)	35.2 (19 – 45)	22 (13 – 45)
Men/women	13/13	6/8
Follow-up duration (months)	32 (12 – 60)	34 (12 – 62)
Prednisone cumulative dose (g)	11.2 (1.2 – 24)**	4.5 (0.9 – 9)
Cyclosporine A cumulative dose (g)	63 (39 –300)**	55 (32 – 98)
Thyroid disorders	14 (53.8%)	5 (35.7%)
Persistent low T3 syndrome[$]	4 (15.4%)	-
*Chronic thyroiditis**	9 (34.6%)	4 (28.6%)
Subclinical hyperthyroidism	4 (15.4)	2 (14.2%)
Subclinical hypothyroidism	2 (7.7%)	-
Thyroid lymphoma	1 (3.8%)	-
Adrenal insufficiency	4 (15%)	-
IGF-I impairment	10 (38%)	1 (7.1%)
Total of patients affected	18 (69%)#	4 (28.6%)

The values are number of patients affected and percentage of the subgroup GVHD. [$]: persistent low T3 syndrome means presence of normal FT3 and FT4 values and TSH levels below the low limit of the normal range at least 12 months after allo-BMT; *: diagnosis of chronic thyroiditis was based on presence of autoantibodies and typical ultrasound pattern; **: $p< 0.001$ *vs* group without GVHD by Mann-Whitney test; #: $\chi 2 = 6.078$; $p = 0.048$ *vs* group without GVHD.

Given the small number of patients, the difference became significant only when disorders were considered altogether (p= 0.048). Men and women were equally affected by chronic GVHD, while older age at BMT and significantly higher cumulative doses of corticosteroids and cyclosporine A (p<0.05, both) were shown in patients affected by chronic GVHD than in patients not affected by this complication. It is difficult to separate clinical effects of immune system damage from those due to immunosuppressive treatments. Thyroid disorders were more frequent in men than in women, but the difference did not reach statistical significance (χ2, 4.945; p=0.08). No significant difference in thyroid, IGF-I and adrenal impairments was found regarding age at BMT (patients transplanted <29 yr and > 29 yr; χ2, 0.44 to 2.25; p=0.32 to 0.09). No influence of acute GVHD occurrence was shown on the endocrine dysfunction. Thyroid disorders occurred also in 35.7% of patients without chronic GVHD.

Discussion

Allogeneic BMT has become a successful treatment for various hematological malignancies and other diseases as well, and is generally performed in young patients with long life expectancy (1,3,7). Several late complications have already been described, but only a few data on risk factors of these long term effects of BMT are available. The disorders are likely attributable to highly aggressive chemotherapy and immune system derangement. While the toxic effects of antiblastic agents have been widely described, the impact of immune system damage on endocrine function is less clear. Allogeneic bone marrow/blood derived stem cell transplantation is the only case of great amount of donor immunological cells infused to a host recognizable as “nonself” to these cells, and thus causing graft-versus–tumor reaction and GVHD. While the first may be beneficial for the eradication of the basic disease, the second represents a serious complication that requires immunosuppressive treatment further compromising the immune system function (9). GVHD may presents as acute or chronic disease based upon the time of onset. Autoantibody formation was more frequently described in the chronic disease, while cellular immunity is usually responsible for the acute forms (9,14,15). Skin, liver and gastrointestinal tract are the tissue mainly affected by the acute form; any tissue can virtually be involved in the chronic disease (13).

The most relevant finding of this study is the high prevalence of endocrine dysfunction in allo-BMT recipients who had not been treated by TBI, even years after BMT. Ovarian failure was found in 95% of women and spermatogenesis damage was likely in 47% of men;

both were considered to be related to alkylating agents administration. On the other hand, thyroid, adrenal and IGF-I impairments were late events occurring in 47.5%, 10% and 27% of all patients, respectively, and were considered to be related to immune system derangement and to immunosuppressive treatment. In particular, the hypothalamus-pituitary-IGF-I axis function has never been investigated in adult allo-BMT recipients, being lower than normal more frequently in patients with chronic GVHD (38%) than in those without this complication (7.1%).

Gonadal failure is the most frequent endocrine consequence of high dose chemotherapy and radiotherapy for any malignancy, and recovery is rare. Loss of the ovarian function is found in almost all women immediately after allogeneic and autologous BMT (5,16-18), as a result of cytotoxic agents and TBI used before transplantation. All alkylating agents have toxic effects on the ovaries (19). High dose therapy damages oocytes and the supporting granulosa cells of both proliferating and resting follicles in a dose-dependent manner (20). In this study, immediate onset of menopause with hypergonadotropic-hypoestrogenic hormone profile was observed in all women but one, who experienced polymenorrhea. Only the youngest woman had spontaneous recovery of ovarian function. According to previous data, age at BMT seems to play an important role, with increased incidence of irreversible ovarian failure with increasing age (21). Premature menopause has serious psychological and medical effects (22) and necessitates treatment. However, HRT can be contraindicated for long periods in case of chronic liver GVHD, or it may not be fully effective because of reduced intestinal or cutaneous absorption, as found in 7 of 21 (30%) women in our cohort. One woman had also permanent complete atrophy of endometrium consequent to BMT.

Forty-seven percent of men had increased FSH levels, suggestive of spermatogenesis damage. Azoospermia was found in all three men tested, one of them with normal FSH levels. This finding suggests that germinal tissue damage can not be excluded by normal gonadotropin values. Unfortunately, only a few men agreed to perform a seminal fluid analysis, and thus sperm parameters were not available in the whole men population. On the other hand, testosterone production was unaffected in our long-term survivors and nobody reported a regression of secondary sexual characteristics. Keilholtz et al. (16) similarly found FSH increase in long term survivors after autologous BMT; in their study however, the prevalence of high FSH levels was higher than in our cohort, likely because our patients did not receive radiation treatment. For development of gonadal failure, age at treatment seems to be less important for men than for women (21). In fact, no difference in the prevalence of high

FSH levels was found according to patient's age in our men cohort. In contrast, underlying disease, type of antiblastic drugs and/or duration of their administration, all affected the likelihood of spermatogenesis damage.

In accordance with previous hypothesis pointing out a relative radio-resistance of the adrenal tissue (7), post-allografting adrenal insufficiency was present even in our cohort of non irradiated patients; it seems related to duration and cumulative dose of corticosteroid treatment received. Autoantibodies to adrenal cortex (ACA, indirect immunofluorescence) and to 21-hydroxylase (immunoprecipitation assay) were assayed in our patients with adrenal insufficiency and were absent. However, some patients with preserved normal function of the adrenal glands had assumed similar cumulative doses for similar periods as patients who experienced adrenal insufficiency. Thus, variable individual sensitivity of the HPA axis to exogenous suppression can by speculated. No stimulation test was performed in the current study; therefore, some minor degree of HPA axis insufficiency could have been overlooked. Indeed, adrenal insufficiency is still an underestimated condition, unless specifically investigated by endocrinologists.

Concerning growth hormone secretion, reduced growth and GH insufficiency has been previously described in children treated by cranial irradiation, but not in those who did not received prophylactic radiotherapy (23). Our results are in keeping with these findings, although GH secretion would have been better investigated by dynamic testing. IGF-I impairment seem to be related to chronic GVHD, that represents a condition of multiorgan injury and is associated with lower BMI. It is well-known that IGF-I reduction is associated with chronic diseases accompanied by conditions as starving or reduced nutrition (25), and this can be the most likely explanation for our results.

Frequent sub-clinical or overt thyroid function impairment has been reported after allo-BMT (4,5,24), less frequently in autologous BMT recipients (16). Increased frequency of transient thyrotoxicosis has been described early after allografting and immune induced injury has been claimed as the pathogenetic factor (4); by contrast, hypothyroidism can be discovered years after transplant and has been ascribed principally to TBI and busulphan therapy (26-28). The prevalence of sub-clinical hyperthyroidism in our patients is in line with data from a recent longitudinal study by Kami et al. (4), and lower than those reported by Toubert et al (5). Since a half of our patients was evaluated for thyroid function only once post-BMT, some transient sub-clinical dysfunction could have been missed. We have observed a case of chronic thyroiditis associated with normal thyroid function, an event unreported till now in allo-BMT recipients. Antibodies toward thyroid were found only in one

patient; however, it but it is known that autoantibodies are not necessarily present in patients with sub-clinical hyper- or hypothyroidism. Low T3 syndrome was present in patients with chronic extensive GVHD even 12-48 months after BMT. This could be due to decreased extra-thyroidal conversion of thyroxin to 3,5,3' triiodothyronin induced by either chronic disease or glucocorticoid treatment (29,30). None of our patients developed overt hypothyroidism probably because they had not been treated by TBI. Although an increase in the prevalence of thyroid papillary carcinoma was found in BMT recipients by two major multicenter studies on late cancer complications (1,31), the only thyroid malignancy in this study was a case of thyroid lymphoma.

A limit of our study is the small sample size, which may have obscured some possible influence of factors different from chronic GVHD on the endocrine dysfunction. Moreover, patients in this study were relatively younger than those reported in other similar studies. Since chronic GVHD is more frequent in older patients, we may have underestimated the real risk of endocrine disorders in an average population of transplanted patients.

While a previous study documented thyroid and adrenal function 5 years after autologous bone marrow/blood-derived progenitor cell transplantation (16), our data suggest that immunosuppressive treatment and immune system derangement play an important role in the development of endocrine dysfunction after allografting. In fact, a high rate of thyroid disorders, adrenal insufficiency and IGF-I decrease was found in patients affected by chronic GVHD. On the other hand, the most frequent endocrine dysfunction was ovarian insufficiency (95%), while spermatogenesis damage was likely in about half of men; gonadal failure is attributed to alkylating agents administration (19,21). Long survival after malignant disease may be associated with endocrine disorders and this is even more relevant for patients undergoing allo-BMT, who are younger than recipients of autologous BMT or other organ transplants. Therefore, these patients deserve life-long attention to detect, prevent and treat symptoms and disorders of endocrine dysfunction. A multidisciplinary management for patients who underwent allogeneic bone marrow-derived hemopoietic stem cells is mandatory.

References

1. Kolb HJ, Socié G, Duell T, Van Lint MT, Tichelli A, Apperley JF, et al. Malignant neoplasms in long-term survivors of bone marrow transplantation. *Ann Intern Med* 1999;131:738-44.

2. Kashyap A, Kandeel F, Yamauchi D, Palmer JM, Niland JC, Moline A, Fung H, et al. Effects of allogenic bone marrow transplantation on recipient bone mineral density: a prospective study. *Biol Blood Marrow Transplant* 2000;6:344-51.

3. Legault L, Bonny Y. Endocrine complications of bone marrow transplantation in children. *Pediatr Transplant* 1999;3(1):60-66.

4. Kami M, Tanaka Y, Chiba S, Matsumura T, Machida U, Kanda Y, et al. Thyroid function after bone marrow transplantation: possible association between immune-mediated thyreotoxicosis and hypothyroidism. *Transplantation* 2001;71(3):406-11.

5. Toubert ME, Sociè G, Gluckman E, Aractingi S, Espèrou H, Devergie A, *et al.* Short- and long-term follow-up of thyroid dysfunction after allogenic bone marrow transplantation without the use of preparative total body irradiation. *British Journal of Haematology* 1997;98:453-57.

6. Schubert MA, Sullivan KM, Schubert MM, et al. Gynecological abnormalities following allogenic bone marrow transplantation. *Bone Marrow Transplant* 1990;5:425-430.

7. Shalet SM, Didi M, Oglivy-Stuart AL, Schulga J, Donaldson MDC. Growth and endocrine function after bone marrow transplantation.[Review] *Clin Endocrinol* 1995;42:333-339.

8. Jadus MR, Wepsic MT. The role of cytokines in graft-versus-host reaction and disease. *Bone Marrow Transplant* 1992;10(1):1-4.

9. Sullivan KM, Agura E, Anasetti C, Appelbaum F, Badger C, Bearman S, et al. Chronic graft-versus-host-disease and other late complications of bone marrow transplantation. *Semin Hematol* 1991;28:250-259.

10. Galimberti M, De Sanctis V, Lucarelli G, Polchi P, Angelucci E, Baronciani D, et al. Endocrine fuction after bone marrow transplantation for thalassemia. *Bone Marrow Transplant* 1991;7(Suppl, 2):74.

11. Glucksberg H, Storb R, Fefer A, Buckner CD, Nieman PE, Clift RA, et al. Clinical manifestation of graft versus host disease in human recipients of marrow from HLA-matched siblings donors. *Transplantation* 1974;18:295-304.

12. Shulman H, Sullivan KM, Weiden PL, McDonald GB, Strikeer GE, Sale GE, et al. Chronic graft-versus-host disease in man: a clinicopathologic study of 20 long term Seattle patients. *Am J Med* 1980;69:204-17.

13. Bostrom L, Ringden O, Jacobson N, Zwaan F, Nilsson B. European multicenter study of chronic graft-versus-host disease. *Transplantation* 1990;49:1100-5.

14. Kier P, Penner E, Bakos S, Kahls P, Lechhner K, Volc-Platzer B, et al. Autoantibodies in chronic GVHD: high prevalence of antinuclear antibodies. *Bone Marrow Transplant* 1990;6:93-6.

15. Siegert W, Stemerowicz R, Hopf U. Antimitochondrial antibodies in patients with chronic graft-versus-host disease. *Bone Marrow Transplant* 1992;10:221-7.

16. Keilholtz U, Max R, Scheibenbogen C, Wüster Ch, Körbling M, Haas R. Endocrine function and bone metabolism 5 years after autologous bone marrow/blood-derived progenitor cell transplantation. *Cancer* 1997;79:1617-22.

17. Schimmer AD, Quatermain M, Imrie K, Ali V, McCrae J, Stewart AK, Crump M, et al. Ovarian function after autologous bone marrow transplantation. *J Clin Oncology* 1998;16:2359-63.

18. Sanders JE, Buckner CD, Amos D, Levy W, Appelbaum FR, Doney K, et al. Ovarian function following marrow transplantation for aplastic anemia or leukemia. *J Clin Oncol*, 1988;6:813-18.

19. Chabner BA, Allegra CJ, Curt A. Antineoplastic agents, in Hardman JG, Limbird LE (eds): Goodman and Gilman's The Pharmacological Basis of Therapeutics (ed 9). New York, NY, McGraw-Hill, 1996, pp 1233-87.

20. Warne G, Fairley KF, Hobbs JB, Martin FIR. Cyclophosphamide-induced ovarian failure. *N Engl J Med* 1973;289:1159-62.

21. Apperley JF. Late complications of transplants in adults. Fertility after high dose therapy. In: Blood and Marrow Transplantation 1998 (The European School of Haematology Handbook 1998) Eds: J Apperley, E. Gluckman, A. Gratwohl Associate Editor: C. Craddock. Paris, pp 151-154.

22. Chiodi S, Spinelli S, Cohen A, Guaalaandi F, Lamparelli T, Lavagetto A, et al. Cyclic sex hormone replacement therapy in women undergoing allogenic bone marrow transplantation: Aims and results. Bone Marrow Transplant 1991;8 (Suppl):47-9.

23. Leiper AD, Stanhope R, Lau T, Grant DB, Blacklock H, Chessells JM, Plowman PN. The effect of total body irradiation and bone marrow transplantation during childhood and adolescence on growth and endocrine function. *British J Haematol* 1987;67:419-26.

24. Benker G, Schäfer U, Herrmanns U, Mahmoud MK, Olbricht T, Schulte HM *et al.* Allogenic bone marrow transplantation in adults: endocrine sequelae after 1-6 years. *Acta Endocrinol*. 1989;120:37-42.

25. Le Roith D, Bondy C, Yakar S, Liu JL, Butler A. The somatomedin hypothesis: 2001. Endocri Rev 2001;22(1):53-74.

26. Cohen A. Late complications of transplants. Endocrinological complications. In: Blood and Marrow Transplantation 1998 (The European School of Haematology Handbook 1998) Eds: J Apperley, E. Gluckman, A. Gratwohl Associate Editor: C. Craddock. Paris, pp 154-159.

27. Boulad F, Bromley M, Black P, Heller G, Sarafoglou K, Gillio A, et al. Thyroid dysfunction following bone marrow transplantation using hyperfractionated radiation. *Bone Marrow Transpl* 1995;15:71-76.

28. Afify A, Shaw PJ, Clavano-Harding A, Cowell CT. Growth and endocrine function in children with acute myeloid leukemia after bone marrow transplantation using busulphan/cyclophosphamide. *Bone Marrow Transpl* 2000;25:253-6.

29. Chopra IJ, Williams DF, Orgiazzi J, Solomon DH. Opposite effects of corticosteroids on serum concentration of 3,3',5 triiodothyronine (reverse T3) and 3,3',5 triiodothyronine (T3). *J Clin Endocrinol Metab* 1975;41:911-20.

30. Chopra IJ, Huang TS, Beredo A, Solomon DH, Chua Teco GN, Mead JF. 1985 Evidence for an inhibitor of extrathyroidal conversion of thyroxin to 3,5,3' triiodothyronine in sera of patients with nonthyroidal illness. *J Clin Endocrinol Metab* 1985;60:666-72.

31. Curtis RE, Rowling RA, Deeg HJ, Shriner DA, Sociè G, Travis LB, et al. Solid cancers after bone marrow transplantation. *N Engl J Med* 1997;336:897-904.

Gonadal status in reproductive age women after stem cell transplantation for hematologic malignancies

Introduction

In recent years, autologous (auto-) and allogeneic (allo-) stem cell transplantation (SCT) have consistently improved disease-free and overall survival of haematological and non-haematological malignancies, and an increasing number of young women can expect to become long-survivors (1). However, improvement in a disease control is associated with significant early and delayed side-effects, one of the most frequent being gonadal damage.

Ovarian failure associated with SCT has been generally ascribed to total body irradiation (TBI) and antiblastic alkylating agents, both widely used for the treatment of haematological malignancies and in conditioning regimens to transplant (2-4). Ovarian damage results in immediate menopause in most women; recovery is rare, being less frequent at older age (3,4). The conditioning regimen aims at eradicating the underlying malignant disease and, in the allogeneic setting, at suppressing the host immune system. Conditioning regimens avoiding TBI, such as the combination of busulphan and cyclophosphamide, have been employed more frequently in the recent years: such a combination, however, still confers a consistent risk for developing ovarian failure, by altering germ cell viability and gonadal hormone production (5-7). Additionally, a "cytokines storm" is released in the peri-transplantation period (8), and a prolonged immune deficiency usually occurs due to delayed and incomplete recovery of the immune system in both allogeneic and autologous SCT recipients.

Allogeneic graft compromises the host immune system more severely than the autologous one due to a prolonged treatment by immunosuppressive drugs needed to avoid the "reverse" rejection. Indeed, acute or chronic graft-versus-host disease (aGVHD, cGVHD) – a complication affecting more than 50% of patients after allografting - induces additional relevant alteration in the immune system (9).

We have recently demonstrated that cGVHD profoundly impairs endocrine functions in allo-STC recipients, even in absence of TBI (10). Moreover, cGVHD was associated with lower sperm count in male long-term survivors after busulphan/cyclophosphamide conditioning (11). It should be emphasized that most previous studies focused on the etiopathogenetic role of

chemotherapy and TBI in inducing ovarian failure and infertility and considered allo- and auto-SCT patients as one group (2,5,6,10-13). Conversely, we observed more frequent recovery of menstrual cycles in women after auto-SCT that in those all-transplanted.

To investigate whether differences in ovarian residual function exist in the allogeneic and autologous setting treated with similar conditioning regimen without TBI, we designed this observational, analytical, prospective, controlled study.

Materials & Methods

Subjects

Fifty consecutive reproductive age women (24 allo- and 26 auto-transplanted, aged 21 to 45 yrs, median 29) who had received SCT for malignant haematopoietic disorders in our institution were enrolled in this pilot study between 12–24 (median, 17) months after SCT. Inclusion criteria were: 1) disease-free at the time of evaluation with complete haematological reconstitution; 2) at least 12 month period elapsed since SCT. Exclusion criteria were: 1) history of radiation therapy, 2) previous history of menstrual cycle disorders or endocrine diseases and 3) previous hormone replacement therapy.

Five women were excluded because of hyperthyroidism (n=1), delayed puberty (n=1), history of oligomenorrhea prior to the disease (n=2) and radiation treatment (n=1). In the remaining 45 patients, primary diseases were acute or chronic myelogenous leukaemia (AML; n=19 and CML; n=7, respectively), acute lymphoblastic leukaemia (ALL; n=4), chronic lymphocytic leukaemia (CLL; n=3), Hodgkin disease (HD; n= 8) and non-Hodgkin lymphoma (NHL; n=4). Allo- and auto-transplanted patients with AML received SCT in their first complete remission, patients with ALL, HD, NHL and CLL were transplanted in second complete remission. Profile at study entry of the patients is summarized in **Table 1**.

Forty-five healthy pre-menopausal women age- and BMI-matched with the patients agreed to participate in this study and were included as controls. All of them had regular menstrual cycles. Exclusion criterion for control women was the use of estrogen/progesterone use as contraceptive or replacement treatment.

Informed consent was obtained by all patients and the study was designed in accordance with the Helsinki II Declaration.

Table 1. Clinical features in 22 women after allogeneic stem cell transplantation (SCT), 23 women after autologous SCT and in 45 healthy women as controls.

	Allo-SCT (n= 22)	**Auto-SCT** (n= 23)	**Controls** (n=45)
Age at evaluation (years)	27.6±2.3	28.2±2.6	28.5±2.4
Time from transplant (months)	18.9±1.2	17.8±1.7	-
BMI (kg/m^2)	23.4±0.9	25±1.0	24.2±0.8
Diagnosis:			
AML	9	10	-
ALL	4	-	-
CML	7	-	-
HD	2	6	-
NHL	-	4	-
CLL	-	3	-
Previous treatment history:			
AML	Idarubicin/mitoxantrone (MIT)/cytarabine (CYT)/etoposide		
ALL	Vincristine/daunorubicin/cyclophosphamide/methotrexate/MIT/CYT/asparaginase		
CML	Hydroxyurea/ interferon-α		
HD	Vincristine/adriblastine/bleomycin/etoposide		
NHL	Methotrexate/adriblastine/cyclophosphamide/vincristine/prednisone		
CLL	Chlorambucil/fludarabine		
Previous use of alkylating agents (n. of patients)	6/22 (27%)	13/23 (56.5%)	
Prednisone cumulative dose (g/m^2)	7.7 ± 1.8	8.2 ± 0.9	-
treatment duration (months)	8.4 ± 1.8*	5.9 ± 0.7	-

Values are expressed as mean ± SEM. Corticosteroid cumulative dose is expressed as prednisone equivalents. Abbreviations: BMI: body mass index; AML: Acute myeloid leukemia; ALL: acute lymphocytic leukemia; CML: chronic myeloid leukemia; HD: Hodgkin disease; NHL: non Hodgkin lymphoma; CLL: chronic lymphocytic leukaemia; *: $p<0.001$ vs. autologous setting

Treatments

Both allo- and auto-transplanted patients for AML, CML and ALL received the same conditioning regimen BUCY2, including busulphan (16 mg/kg in 4 days) and cyclophosphamide (120 mg/kg in 2 days). Patients with HD, NHL, and CLL were conditioned with the BEAM protocol, consisting of carmustine (300 mg/m^2 in 1 day), etoposide (200 mg/m^2 in 4 days), cytarabine (400 mg/m^2 in 4 days) and melphalan (140 mg/m^2 in 1 day). Allo-SCT recipients received bone marrow-derived stem cells from HLA-identical siblings. After allografting, aGVHD prophylaxis was performed with cyclosporin A (1 mg/kg i.v. from day –1 to +21, then 10 mg/kg p.o. for 6 months) and short course methotrexate (10 mg/kg for 4 doses). Acute GVHD was treated by high-dose methylprednisolone (2-10 mg/kg for 10 days), followed by slow dose tapering in the following 6 months, monitoring patients' clinical conditions. Chronic GVHD was treated by prednisolone at doses of 1-2 mg/kg, associated with CsA at doses ranging from 1 to 8 mg/kg/day. Previous treatment history included alkylating agents for patients with ALL, CLL, HD and NHL (Table 1) and consisted in a mean 6-month duration of chemotherapy for all diagnoses. Acute GvHD was considered according to the Glucksberg grading system (14), and cGVHD was graded as either limited or extensive, based upon clinical severity and target organs involvement (15). Nine women had been affected by aGVHD of global grade 1-3 and 13 women were affected by cGVHD (six limited and seven extensive form). Three women with the limited form had liver involvement and other three skin manifestation. Liver, skin, intestine and eyes were the most common cGVHD sites in the extensive form with variable combination (liver and gastrointestinal [n=2], liver and eyes [n=2], skin, gut, and eyes [n=2], skin, gut, and kidney [n=1]. Liver manifestation included an increase of liver enzymes in all women (ALT: 50-1100 U/L; AST: 82-761 U/L; γ-glutamyl-transferase: 90-350 U/L) and reversible cholestasis with alkaline phosphatase (487-785 U/L) and bilirubin increase (5 -9 mg/dl) associated with icterus in two. Skin lesions included lichen planus and focal epidermal atrophy. Ocular dryness (Sjogren-like syndrome) was the major ophthalmologic manifestation, while oral mucosa dryness and lichenoid lesions were the upper gastrointestinal tract symptoms. Diarrhoea and malabsorption were present in two women. One woman developed reversible membranous glomerulonephritis with nephrotic syndrome. All these patients were treated as stated above for a period ranging 6-24 months (**Table 1**). After evaluation, hormone substitutive treatment with estradiol and medroxyprogesterone was initiated in all women with amenorrhea unless contraindicated or refused.

Study design

Previous medical records were reviewed to obtain complete information on patients' outcome, including data on menstrual history pre-transplantation. Post-transplantation data were recorded prospectively. Body-mass index (BMI, kg/m^2) was determined in all subjects. In the control group the endocrine and ultrasonography was performed in the early follicular phase of the menstrual cycle (2-3 day).

Endocrine evaluation

In all subjects, blood samples were obtained between 08:00 and 10:00 a.m. Circulating FSH, LH, prolactin, 17-β-estradiol, testosterone and Δ_4-androstenedione levels were assayed to assess the hypothalamic-pituitary-ovarian function. Dehydroepiandrosterone sulphate (DHEAS) was also measured as the major androgen of adrenal origin. All measurements were performed by commercially available kits: 17-β-estradiol by RIA (Nichols Institute Diagnostics, San Juan Capistrano, CA), total testosterone and DHEAS using Immulite, solid phase chemiluminescent enzyme immunoassay (DPC, Los Angeles, CA, USA); FSH and LH with RIA Biodata S,p.A. kits (Rimini, Italy); androstenedione using RIA Diagnostic Systems Laboratories kit (Webster, TX, USA); at least 2 different samples were taken in all subjects and the average value was calculated for each hormone. Detection limits were 3 pg/ml for 17-β-estradiol, 5 ng/dl for testosterone, 10 ng/dl for Δ4-androstenedione and 2 μg/dl for DHEAS. Intra-assay and inter-assay coefficients of variation were below 7% and 15%, respectively, for all endocrine determinations.

Ultrasonographic evaluation

Pelvic ultrasonography was performed in all patients and controls using a 7.5-MHz transvaginal transducer. The study was performed by a single well-trained operator (S.P.), blind in respect to patient or control exam. Ovarian and uterine volumes were estimated using the ellipsoid formula (longitudinal x antero-posterior x transversal diameter x 0.523) (16). The mean ovarian volume for each woman was calculated. Number of follicles and endometrium thickness were also recorded.

Statistical analysis

Student's t test for paired and unpaired data was used to compare the group of patients and controls, and different subgroups of patients, respectively. The nonparametric Mann-Whitney U test was used to compare hormone levels in patients and controls as well as in allo- *vs.* auto-SCT

patient group, when Wilk-Shapiro's test was not consistent with the Gaussian distribution of the data. To assess the dependence between endocrine and ultrasonographic features of the patients, the Pearson's correlation coefficient was calculated. As possible predictor variables of menstrual cycle recovery were considered age at transplant, BMI, time from SCT, previous use of alkylating agents and type of transplant. Univariate and regression analysis was performed using the cycle recovery as a binary outcome variable. Two-sided P values <0.05 were taken as statistically significant. Data are expressed as mean ± SEM.

Results

Menstrual history data indicated that the disappearance of menstrual cycles was caused by the myeloablative conditioning therapy in 30 women, while other 15 had amenorrhea after conventional chemotherapy shortly before SCT. Recovery of menstrual cycles was slightly, but not statistically, more frequent in the group of auto-transplanted patients (4/23 *vs*. 2/22 in the allo-SCT group). Women who experienced recovery of the ovarian function were younger than those who did not, and were transplanted from a longer period (p<0.05). BMI, corticosteroid cumulative dose and duration of treatment did not differ in women with or without cycle recovery (**Table 2**).

Table 2. Univariate predictors of cycle recovery

	Cycle recovery	**No cycle recovery**	***P***
Age at SCT (years)	21.7± 2.3	28.5 ± 2.5	0.013
BMI at SCT (kg/m^2)	23.6±1.6	23.9±0.9	NS
BMI at evaluation (kg/m^2)	22.8 ± 1.8	24.7± 1.4	NS
Corticosteroid dose (mg/m^2)	8.9 ± 2.7	8.1 ± 1.1	NS
Corticosteroid duration (mos)	9.6 ± 2.2	7.3 ±1.9	NS
Time from SCT (mos)	20.2 ± 1.7	17.1 ± 1.8	0.047
Patients previously treated with alkylating agents (n./total)#	3/6 (50%)	16/39 (41%)	NS

BMI: body mass index; SCT: stem cell transplantation, corticosteroid dose was calculated as prednisone equivalents**.** Students' *t*-test was used to compare mean values; # $\chi 2$ test was used for comparison of the frequency.

By the logistic regression, however, only young age at SCT was a positive predictor of cycle recovery ($p<0.05$), while previous use of alkylating agents, type of transplant, BMI and time elapsed from transplant did not reach statistical significance (**Table 3**).

Table 3. Predictors of cycles recovery by regression analysis

	Odds ratio (95% C.I.)	***P***
Age at SCT <21 yrs	6.8 (0.191 - 0.829)	<0.05
Allogeneic setting	0.47 (-0.337 – 0.116)	NS
Previous alkylating treatment	0.72 (-0.303 - 0.143)	NS
Time from SCT > 18 months	2.1 (-0,116 - 0,336)	NS

SCT: stem cell transplantation

At study entry, the groups of allo- and auto- transplanted women had similar age and follow-up period after transplantation, while they differed in the underlying haematological disease. Patients with AML and CML were not treated by alkylating agents, all patients with CML received allogeneic SCT, while patients with AML were treated by allo- or auto-SCT in a similar proportion (9 *vs*. 10). Consequently, before transplant alkylating agents were more frequently used in the auto-SCT group. The dose of corticosteroids was similar in both patient groups, although most allo-transplanted patients were treated for longer periods, and treatments were stopped more recently, as in the auto-SCT group steroid treatment took a part of chemotherapeutic regimens previous to the transplant (**Table 1**).

Endocrine evaluation

Endocrine parameters of the two patients' groups and controls are shown in **Table 4.** Serum gonadotropin levels were significantly higher ($P<0.001$) while 17-β-estradiol, Δ_4-androstenedione, testosterone ($P<0.001$) and DHEAS ($P<0.05$) were lower in allo-SCT patients than in controls. The auto-SCT women had significantly higher ($P<0.001$) gonadotropin values and lower 17-β-estradiol ($P<0.001$) than controls while Δ_4-androstenedione and testosterone levels were similar. Serum gonadotropin levels were significantly higher ($P<0.001$) and 17-β-estradiol, Δ_4-androstenedione and testosterone significantly lower ($P<0.05$) in the allo-SCT compared to the auto-SCT group. Prolactin levels were within the normal range in all subjects, without any difference between patients and controls. Similar results with the same statistical significance were obtained when only women affected by AML were compared (10 auto- and 9 allo-

transplanted, data not shown). When allo-SCT patients were divided according to presence of cGVHD, ovarian failure resulted to be slightly more severe in women with cGVHD (**Table 5**).

Ultrasonographic evaluation

Ultrasonographic results in the patients' groups and in controls are shown in **Table 4.** Ovarian size in our control group was within the normal range of pre-menopausal women with similar age (16-18). Volumes of ovaries and uterus were lower in the patients, both allo- and auto-transplanted, when compared to the controls ($P<0.001$). Furthermore, either uterine or ovarian volumes were significantly ($P<0.001$) smaller in the allo- than in the auto-SCT group.

In the patient group, the mean number of follicles resulted significantly ($P<0.0001$) lower in comparison with controls; and allo- transplanted women had less follicles than those auto-transplanted ($p<0.0001$). In particular, 4 to 12 small and larger follicles (diameter, ≤12 mm) per ovary were observed in the control group, several small ovarian follicles (diameter ≤6 mm) were found in 12 women after auto-SCT and in only four after allo-SCT. In the allo- and auto-SCT groups, without difference between groups, the mean endometrial thickness were significantly ($P<0.05$) lower in comparison with controls (**Table 4**). In fact, endometrial thickness was very low or undetectable in most SCT patients.

Table 4. Endocrine and ultrasound findings in women after allo- or auto- stem cell transplantation (SCT) and in controls.

	Allo-SCT (n= 22)	***P*** *vs*. controls	**Auto-SCT** (n= 23)	***P*** *vs.* controls	**Controls** (n=45)	**Reference intervals**
FSH	127±16[b]	<0.001	63.6±7.9	<0.001	6.8±3.2	2-13 U/L
LH	75±10b	<0.001	28.2±3.7	<0.001	10.6±2.6	2-15 U/L
17-β-estradiol	48.8±6.7[a]	<0.001	73.05±8.8	<0.001	183.6±19.2	140-734 pmol/L
Testosterone	0.55±0.07[a]	<0.001	1.32±0.14	0.551	1.35±0.14	0.68-2.8 nmol/L
Δ_4-Androstenedione	1.74±0.14 [a]	<0.001	3.84±0.17	0.947	4.1±1.22	3.4-10 nmol/L
Dehydroepiandrosterone sulphate	3.3±0.29	<0.05	4.3±0.7	NS	4.7±0.3	1.4-7 µmol/L
Prolactin	8.8±0.89	NS	9±1.2	NS	9.5±1.3	2-15 µg/L
Ovarian volume (cm^3)	1.35±0.14[c]	<0.001	3.3±0.14	<0.001	8.2±0.17	5.7-10 cm^3 *
Uterine volume (cm^3)	27.2±0.76[c]	<0.001	47.3±1.56	<0.001	53.0±1.9	-
N° of follicles per ovary	0.4±0.7 [c]	<0.001	3±2	<0.001	8±2	-
Endometrial thickness (mm)	3.5±0.15	<0.05	3.9±0.14	<0.05	6.4±0.16	5-8 mm*

Values are expressed as mean ± SEM. Ovarian and uterine volumes were calculated according to the ellipsoid formula (multiplication of 3 diameters x 1/3π). *: data relative to the early follicular phase of menstrual cycle. NS: not significant; a: *P*<0.05, b: *P*<0.01 and c: *P*<0.001 *vs.* auto-SCT.

According to the presence of cGVHD, ovarian and uterine volumes were significantly lower in women with than in those without GVHD (P<0.001; **Table 5**).

Table 5. Clinical features, endocrine and ultrasound evaluation in women after allogeneic SCT according to chronic GVHD occurrence.

	Chronic GVHD (n= 13)	**No chronic GVHD (n= 9)**	***P***
Age (years)	26.6 ±3.1	25.2 ±3.8	NS
Time since transplant (mos)	18.5 ± 1.1	19 ± 1.3	NS
BMI (kg/m^2)	21.2 ± 2.2	24.1 ± 1.9	NS
FSH (IU/L)	140 ± 22.3	106.3 ± 6.6	<0.05
LH (IU/L)	78.6 ± 12.5	66.3 ± 2.4	NS
17-β-estradiol (pmol/L)	37.4 ± 4.26	58 ± 5.1	<0.05
Prolactin (μg/L)	7.65 ± 1.4	7.9 ± 1.5	NS
Testosterone (nmol/L)	0.4 ± 0.1	0.55 ± 0.11	NS
Δ_4-androstenedione (nmol/L)	1.57 ± 0.17	1.85 ± 0.23	NS
DHEAS (μmol/L)	2.7 ± 0.32	3.5 ± 0.23	<0.05
Ovarian volume (cm^3)	1.05 ± 0.14	1.4± 0.15	<0.001
Uterine volume (cm^3)	23.1 ± 0.9	34± 1.9	<0.001

The values are expressed as mean ± SEM. GVHD: graft-vs-host disease; BMI: body mass index; DHEAS: dehydroepiandrosterone sulphate; Ovarian and uterine volumes were calculated according to ellipsoid formula (multiplication of 3 diameters x 1/3π).

As expected, in the patient group considered as a whole, 17-β-estradiol (r= 0.51; P<0.05) and Δ_4-androstenedione (r= 0.55; P<0.05) values correlated with ovarian volume, 17-β-estradiol was also inversely correlated with FSH levels (r= —0.67; p<0.05). Uterine volume correlated with ovarian volumes (r=0.77; P<0.01). On the other hand, no correlation was found between age, BMI, time elapsed from transplantation and endocrine or ultrasonographic parameters.

Discussion

The results of the present study show that reproductive age women undergoing allogeneic or autologous SCT have a premature ovarian failure (secondary hypergonadotropic amenorrhoea), that persists up to 24 months after transplant in most cases (87%). Only younger age (<21 yrs) at SCT was an important predictor of ovarian function recovery. Although cycles recovered more

frequently in auto-SCT women, the difference when compared with the allo-SCT group was not significant. The longer period elapsed between SCT and evaluation in women who experienced recovery of ovarian function, suggests that recovery is not an early event and may occur even long time after SCT. By regression analysis, the type of transplant, BMI, previous use of alkylating agents and steroids were not associated with a different probability of spontaneous recovery of ovarian function. However, some of these factors can become significant in larger populations of these peculiar patients. In fact, age, weight gain, disease stage and use of systemic chemotherapy resulted important predictors of menopause onset also in other female neoplastic populations (19).

Ovarian failure is reported to be a rather frequent event after myeloablative therapy followed by SCT in postpubertal patients (3,4,6,11,12). However, whether it is due to high-dose alkylating agents and/or TBI used in the conditioning regimens, to conventional chemotherapy before myeloablative treatment, to the combination of all three treatment approaches (2,20) or to other unknown factors is still to be fully elucidated. Most previous studies (2,5,6,10-13), having as endpoints spontaneous recovery of ovarian function, infertility or pregnancy rates, have focused on the role of different treatments, such as TBI and chemotherapy, in inducing ovarian failure. Additionally, in previous studies no distinction between allo- and auto-SCT subjects has been performed, and different conditioning regimens, also including TBI, were considered altogether. In the attempt to overcome these limitations, we included patients receiving a similar conditioning treatment, excluding TBI, and analyzed auto- and allo-SCT patients also separately.

One of the relevant finding of the current study, is that allo-transplanted recipients had lower 17-β-estradiol and androgen circulating levels and lower ovarian volume than in the auto-transplanted ones. Importantly, among allo-transplanted women those who developed cGVHD had lower 17-β-estradiol, Δ_4-androstenedione, testosterone and DHEAS and higher FSH and LH levels than those who did not develop GVHD. The potential role of a lower BMI (indicating less fat mass after cGVHD) in inducing androgen aromatization to estrogens seems to be negligible, considering the lower circulating androgen levels and the absence of correlation between BMI and endocrine parameters. It should also be noted that auto-transplanted women had an ovarian volume similar to that observed in women after a corresponding interval from physiological menopause; in contrast the ovarian volume of allo-SCT was lower (16-18).

The differences in endocrine and ultrasound parameters between auto- and allo-transplanted patients were similar in the subgroup including only women with AML, indicating that results did not depend on the underling disease, but likely on different transplant procedures. Chemotherapy is known to induce ovarian failure by apoptotic changes in pre-granulosa cells causing follicle loss (21,22). In particular, alkylating drugs have been shown to alter base pairs,

leading to DNA cross-links and single-strand breaks (23). The combination of high-dose cyclophosphamide and busulphan is one of the most potent conditioning regimens often inducing ovarian failure (24). Primordial follicles were absent in some patients at ovarian biopsy, likely due to damage of oocytes and proliferating as well as resting follicles supporting granulosa cells (22). Additional finding in our cohort of patients was that, beside having lower 17-β-estradiol levels, allo-SCT women had lower testosterone levels than both auto-SCT and control women. The ovarian secretion of estrogens is reported to decline faster than that of androgens after physiological menopause, likely due to progressive follicular athresia with persistent androgen production from stromal ovarian tissue (25). Consequently, the ovaries were hypothesized to become primarily androgen-producing glands, able of maintaining gonadotropin responsiveness for many years (25). As a matter of fact, different studies had shown that ovarian testosterone production remains relatively constant, thereby increasing the relative ovarian contribution to the global testosterone production and androgen-to-oestrogen ratio (26,27). Conversely, absent gonadotropin receptors and insignificant 17-β-estradiol and androgen production has been recently shown in post-menopausal women after cessation of menstrual cycles for at least 5 years (28). The different period of time elapsing from menopause onset to evaluation could explain the difference between these apparently contrasting findings. Serum 17-β-estradiol, Δ_4-androstenedione and testosterone levels in our cohort of allo-SCT patients were significantly decreased, indicating both follicles and stromal endocrine cells damage, with signs of more severe damage in women affected by cGVHD. Conversely, in auto-SCT patients only serum 17-β-estradiol levels were decreased, while Δ_4-androstenedione and testosterone levels were normal 12-24 months after transplantation. The ovarian contribution to lower serum androgens is confirmed by the correlation between ovarian volume and 17-β-estradiol and androgen levels. However, since lower DHEA-S values were found in allo-SCT patients, particularly in those with cGVHD, effects of longer and more recent corticosteroid use on adrenal androgen secretion can not be ruled out. The degree of immune system involvement might be hypothesized as major difference between the two groups.

While the toxic effects of antiblastic agents have been widely described as detrimental, the impact of immune system damage on endocrine function in transplanted women is not clear. Allogeneic SCT is an exceptional condition in terms that a massive amount of donor immunological cells is infused to a host, and they can recognize the host as “non-self”. The best known target organs for such a “graft versus host reaction” are skin, liver, gut and lung, but any other organ can be targeted including gonads, and the neoplastic tissue itself. The prophylaxis and treatment of GVHD require immuno-suppressive drugs, which further compromise immune system function and whose effects can not be separate from those of the immune system

dysregulation itself. Azoospermia related to cGVHD (10) was also reported in allo-SCT recipients, in line with our hypothesis on a possible involvement of the immune system derangement in the gonadal damage.

In the current study, we did not investigate circulating autoantibodies against ovary since the existence of a correlation between anti-ovarian antibody titre, cellular immune dysfunction and histological evidence of inflammation in women with autoimmune ovarian failure is still undefined. Circulating anti-ovary autoantibodies are not correlated with clinical activity of autoimmune oophoritis and their pathogenetic role remains questionable (29,30).

In conclusion, the results of the current study indicate that after autologous SCT ovaries have characteristics similar to those occurring after a similar period of physiological menopause, whereas allogeneic SCT is associated with a more severe injury, characterized by lower estradiol and androgen levels, and ovarian size. Chronic GVHD following allografting further worsen this condition. Ovarian failure in SCT recipients seems to be related principally to the myeloablative conditioning regiments, however, ovarian steroid levels and size can be further influenced in the allogeneic setting, likely by a major deregulation of the immune system and its treatments. The impact of a lower residual oestrogen and androgen steroid secretion after allo-transplantation on women's health, especially in terms of osteoporosis, cardiovascular risks and quality of life remains to be established as well as any future potential therapeutic implication and pregnancy outcomes (31).

References

1. Kolb HJ, Socié G, Duell T, Van Lint MT, Tichelli A, Apperley JF, Nekolla E, Ljungman P, Jacobsen N, van Weel M, *et al.* Malignant neoplasms in long-term survivors of bone marrow transplantation. *Ann Intern Med* 1999; **131:** 738-744.

2. Couto-Silva AC, Trivin C, Thibaud E, Esperou H, Michon J, Brauner R. Factors affecting gonadal function after bone marrow transplantation during childhood. *Bone Marrow Transplant* 2001; **28:** 67-75.

3. Schubert MA, Sullivan KM, Schubert MM, Nims J, Hansen M, Sanders JE, O'Quigley J, Witherspoon RP, Buckner CD, Storb R, *et al.* Gynecological abnormalities following allogenic bone marrow transplantation. *Bone Marrow Transplant* 1990; **5:** 425-430.

4. Shalet SM, Didi M, Oglivy-Stuart AL, Schulga J, Donaldson MDC. Growth and endocrine function after bone marrow transplantation. *Clin Endocrinol* 1995; **42:** 333-339.

5. Afify A, Shaw PJ, Clavano-Harding A, Cowell CT. Growth and endocrine function in children with acute myeloid leukemia after bone marrow transplantation using busulphan/cyclophosphamide. *Bone Marrow Transplant* 2000; **25:** 1087-1092.

6. Sanders JE, Buckner CD, Amos D, Levy W, Appelbaum FR, Doney K, Storb R, Sullivan KM, Witherspoon RP, Thomas ED. Ovarian function following marrow transplantation for aplastic anemia or leukemia. *J Clin Oncol* 1988; **6:** 813-818.

7. Sklar CH, Boulad F, Small T, Kernan N. Endocrine complications of pediatric stem cell transplantation. *Front Biosc* 2001; **6**: 17-22.

8. Jadus MR, Wepsic MT. The role of cytokines in graft-versus-host reaction and disease. *Bone Marrow Transplant* 1992; **10:** 1-4.

9. Sullivan KM, Agura E, Anasetti C, Appelbaum F, Badger C, Bearman S, Erickson K, Flowers M, Hansen J, Loughran T, *et al.* Chronic graft-versus-host-disease and other late complications of bone marrow transplantation. *Semin Hematol* 1991; **28:** 250-259.

10. Tauchmanovà L, Selleri C, De Rosa G, Pagano L, Orio F Jr, Lombardi G, Rotoli B, Colao A. High prevalence of endocrine dysfunction in long-term survivors after allogeneic bone marrow transplantation for hematological diseases. *Cancer* 2002; 95: 1076-1084.

11. Grigg AP, McLachlan R, Zaja J, Szer J. Reproductive status in long-term bone marrow transplant survivors receiving busulphan-cyclophosphamide (120 mg/kg). *Bone Marrow Transplant* 2000; **26:** 1089-1095.

12. Chatterjee R, Goldstone AH. Gonadal damage and effects on fertility in adult patients with haematological malignancy undergoing stem cell transplantation. *Bone Marow Transplant* 1996; **17:** 5-11.

13. Schimmer AD, Quatermain M, Imrie K, Alì V, McCrae J, Stewart AK, Crump M, Derzko Ch, Keating A. Ovarian function after autologous bone marrow transplantation. *J Clin Oncol* 1998;**16:** 2359-2363.

14. Glucksberg H, Storb R, Fefer A, Buckner CD, Neiman PE, Clift RA, Lerner KG, Thomas ED. Clinical manifestation of graft versus host disease in human recipients of marrow from HLA-matched siblings donors. *Transplantation* 1974; **18:** 295-304.

15. Shulman H, Sullivan KM, Weiden PL, McDonald GB, Striker GE, Sale GE, Hackman R, Tsoi MS, Storb R, Thomas ED. Chronic graft-versus-host disease in man: a clinicopathologic study of 20 long term Seattle patients. *Am J Med* 1980; **69:** 204-217.

16. Pavlik EJ, De Priest PD, Gallion HH, Ueland FR, Reedy MB, Kryscio RJ, van Nagell JR Jr. Ovarian volume related to age. *Gynecol Oncol* 2001; **80:** 333-334.

17. Flaws JA, Rhodes JC, Langenberg P, Hirshfield AN, Kjerulff K, Sharara FI. Ovarian volume and menopausal status. *Menopause* 2000; **7:** 53-61.

18. Wehba S, Fernandes CE, Ferriera JA, Azevedo LH, Machado RB, Lunardelli JL, Lima SR, Iwamato V. Tansvaginal ultrasonography assessment of ovarian volumes in postmenopausal women. *Rev Paul Med* 1996; **114:** 1152-1155.

19. Goodwin PJ, Ennis M, Pritchard KI, Trudeau M, Hood N. Risk of menopause during the first year after breast cancer diagnosis. *J Clin Oncol* 1999; **17:** 2365-2370.

20. Keilholtz U, Max R, Scheibenbogen C, Wüster Ch, Körbling M, Haas R. Endocrine function and bone metabolism 5 years after autologous bone marrow/blood-derived progenitor cell transplantation. *Cancer* 1997;**79:** 1617-1622.

21. Chabner, B.A., Allegra, C.J., Curt, A. (1996) Antineoplastic agents. In Hardman, J.G. and Limbird, L.E. (eds) *Goodman and Gilman's The Pharmacological Basis of Therapeutics* (ed 9). New York, NY, McGraw-Hill, pp. 1233-1287.

22. Warne G, Fairley KF, Hobbs JB, Martin FIR. Cyclophosphamide-induced ovarian failure. *N Engl J Med* 1973; **289:** 1159-1162.

23. Epstein RJ. Drug-induced DNA damage and tumor chemosensitivity. *J Clin Oncol* 1990;**8:** 2062-2084.

24. Brennan BMD, Shalet SM. Endocrine late effects after bone marrow transplant. *Br. J Haematol* 2002; **118:** 58-66.

25. Lindgren R, Gunnarsson C, Jakobsson A, Hammar M. Hypersecretion of ovarian androgens may be gonadotropin dependent many years after menopause. *Maturitas* 2000; **34:** 43-46.

26. Adashi EY. The climacteric ovary as functional gonadotropin-driven androgen producing gland. *Fertil Steril* 1994; **62:** 20-27.

27. Judd HL, Judd GE, Lucas WE, Yen SSC. Endocrine function of the postmenopausal ovary. Concentration of androgens and estrogens in ovarian and peripheral vein blood. *J Clin Endocrinol Metab* 1974; **39:** 1020-1024.

28. Couzinet B, Meduri G, Lecce MG, Young J, Brailly S, Loosfelt H, Milgrom E, Schaison G. The postmenopausal ovary is not a major androgen-producing gland. *J Clin Endocrinol Metab* 2001; **86:** 5060-5066.

29. Hoek A, Schoemaker J, Drexhage HA. Premature ovarian failure and ovarian autoimmunity. *Endocr Rev* 1997; **18:** 107-134.

30. Kinch RAH, Plunkett ER, Smout MS, Carr DH. Primary ovarian failure: a clinicopathological and cytogenetic study. *Am J Obstet Gynecol*(1965; **91:** 630-641.

31. Sanders JE, Hawley J, Levy W, Gooley T, Buckner CD, Deeg HJ, Doney K, Storb R, Sullivan K Witherspoon R. Pregnancies following high dose cyclophosphamide with or without high-dose busulphan or total-body irradiation and bone marrow transplantation. *Blood* 1996; **87:** 3045-3052.

Estrogen-progestogen induced hematocolpometra following allogeneic stem cell transplant

Introduction

The most frequent and obvious complication of the reproductive system after allogeneic stem cell transplantation (allo-SCT) is ovarian failure, which occurs in over 90% of female patients (1,2). While ovarian toxicity depends on age, type of transplant, conditioning regimens, previous use of alkylating agents and radiation treatment (1), some other gynecological complications have been described in association with extensive chronic graft-versus-host disease (cGVHD). In particular, propensity to develop vulvo-vaginal infection, vaginal and cervical stenosis, and disfigurement of the internal and external genitalia, sometimes with peritoneal involvement, have been reported. All of these conditions may negatively influence sexual function, causing dyspareunia, reproductive failure and difficult labor (2-4). Chronic GVHD is a late complication after allo-SCT occurring in up to 50% of patients. Although skin, liver, eyes and the gastrointestinal system are most frequently involved, hypothetically any organ can be targeted by this disorder. Gynecological manifestation is relatively rare, but probably underestimated. Corson *et al*. have described a 10% prevalence of vulvovaginal lesion in the Seattle population, when systematically evaluated, including 41 women with cGVHD (4).

As women undergoing allo-SCT are prevalently young (young age is one of the selection criteria), giving them estrogen + progestogen hormonal therapy (EPT) becomes mandatory in order to prevent the multiple negative effects of premature menopause. It should be underlined that, regardless sexual activity of the women, gynecological complications may become manifest after the introduction of EPT.

Methods and Results

We here describe our experience concerning gynecological complications in a population of 30 allo-transplanted women who were followed in a single center 12-120 months after SCT. Their median age at transplant was 32 years (range, 12-40). All women

had been conditioned by " BUCY 2" regimen including busulphan (16 mg/kg in 4 days) and cyclophosphamide (120 mg/kg in 2 days) and nobody had received any radiation therapy. The reasons for transplant included acute myeloid leukemia (n=10), acute lymphocytic leukemia (n= 6), chronic myeloid leukemia (n=12) and severe aplastic anemia (n=2). Fourteen women had been or were still affected by cGVHD: 6 by the limited form and 8 by the extensive one.

Three women were found to have gynecological manifestation of cGVHD that became evident shortly after HRT introduction. Following are the reports of single cases:

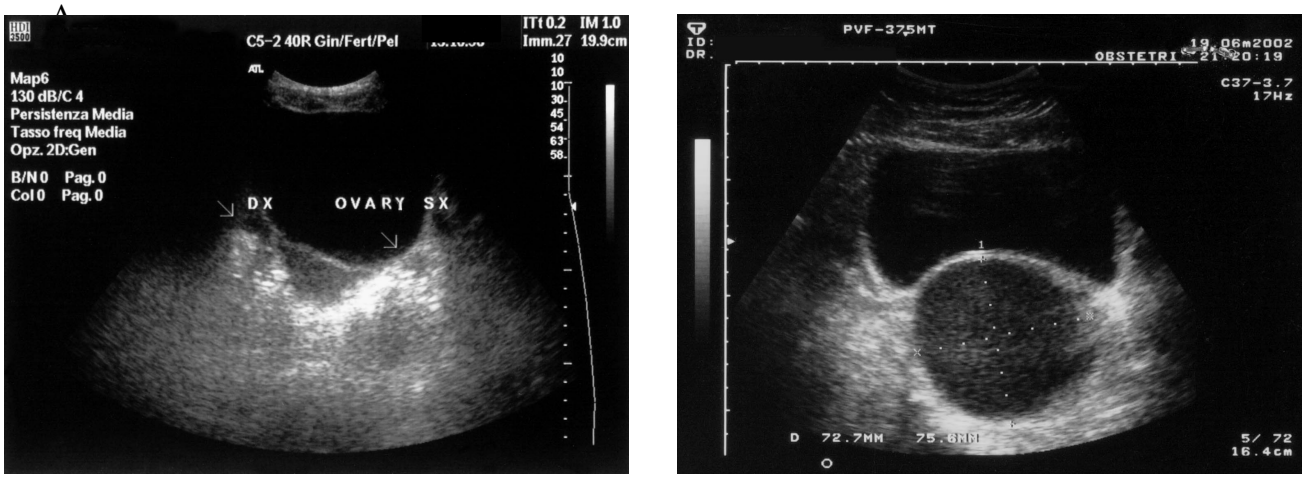
Case 1

A 31-year old woman with acute myeloid leukemia had received SCT from a HLA identical brother in her first complete remission. She had had two previous pregnancies terminated with a cesarean section. Acute GVHD was mild and only dermal. On the other hand, extensive cGVHD involved multiple organs causing dermal, bucal (lichenoid lesions) and ophthalmic (Sjögren-like) lesions. Hypergonadotropic amenorrhea followed SCT and sequential EPT was introduced 18 months after allografting. The patient was married, sexually active, disease-free and had no sign of active cGVHD at the moment of EPT introduction. Cyclic withdrawal bleeding was mild and after the third cycle she complained of pelvic pain and dyspareunia. Gynecological examination revealed vulvar atrophy, a large, palpable and painful mass, and cervical adhesions. Transabdominal ultrasonography revealed the presence of hematocolpos. EPT was interrupted, cervical synecchiae were freed digitally and about 250 ml old blood was evacuated from uterus. Local treatment with equine conjugated estrogens (ECE) was continued for 6 months. Thereafter, when there was no evidence of cervical or vaginal adherence, she initiated sequential EPT again; a recent transvaginal ultrasonography (TV-US) has not detected any abnormal findings.

Case 2

A 24-year old patient (para 0) affected by chronic myelocytic leukemia received SCT from her brother in the first remission. After 15 months, she relapsed and received two lymphocyte infusions. Thereafter, she developed extensive cGVHD with liver, dermal (sclerodermatous lesions) and ophthalmic manifestation. She was surgically treated for obstruction of the lachrymal canal. Three years after SCT, she suffered from severe menopausal symptoms, osteoporosis and had no sign of active cGVHD. Sequential EPT

was initiated with very mild withdrawal bleeding. During the third treatment cycle she referred to a gynecologist because of a progressively increasing pelvic pain.



A. B.

Figure 1. Pelvic ultrasonography before EPT introduction (panel A) and 3 months later when hematocolpometra was diagnosed (panel B)

A hematocolpometra was revealed by ultrasonography (**Figure 1**) and treated during hysteroscopy in general anesthesia with digital lysis of multiple vaginal and uterine synecchiae, evacuating approximately 300 ml of old blood. Thereafter, local therapy with ECE was initiated with improvement of the picture. However, the patient reported that the adhesion tended to reoccur whenever she interrupted the treatment. Four months after hysteroscopy, a continuous EPT was introduced, under strict monitoring of the patient's condition, continuing also the local treatment.

Case 3

A 22-year old (gravida 2 para 2) patient with chronic myeloid leukemia received allo-SCT from a HLA-identical sister. She developed acute liver GVHD and veno-occlusive like disease, continuing as severe cGVHD at the same site. Eleven months after SCT she was treated with decompression surgery because of bilateral femoral head avascular necrosis. Because of severe menopausal symptoms consequent to SCT, she started a sequential EPT 26 months after grafting. No withdrawal bleeding occurred at the end of the first cycle. At the gynecological examination, an extensive vulvar atrophy was found, with quite complete vaginal obliteration. EPT was withdrawn and vaginal dilatators with local application of ECE were prescribed to maintain vaginal patency. When asked,

the patient, although married, reported no sexual activity after SCT. Improvement but not the disappearance of local symptoms was observed after 2, 4 and 16 weeks of local treatment.

Other findings

Another woman affected by chronic myeloid leukemia experienced no withdrawal bleeding with cyclic EPT despite otherwise normal gynecological examination. Her gonadotropin values decreased from post-menopausal levels to the normal range and vasomotor symptoms disappeared, findings that indicated satisfactory absorption of hormonal treatment. However, the woman had no evidence of endometrium at TV-US repeated at different periods of sequential EPT cycles. Asherman's syndrome consequent to chemotherapy was diagnosed and EPT has been resumed. She has no history of acute or cGVHD.

Risk factors for gynecological disorders

Three out of 30 allo-transplanted women followed up in our center had severe involvement of gynecological organs. The prevalence was 3 out of 14 patients affected by cGVHD. Chronic GVHD with dermal and mucosal localization was present in two women while the third had only liver involvement (**Table 1**). The age at SCT did not differ between women affected or not by the stenosis. None of our patient had a post-transplant history of infection, suggesting no infectious etiology of the dystrophic process observed. Fluids from evacuated uteri were cultured and resulted negative for Gram-positive or Gram-negative bacteria. Chronic GVHD seems to be the most plausible cause of gynecological disorders in the three patients described above.

Discussion

Quite a high percentage (3/14) of allo-transplanted women with cGVHD followed up in our center had severe involvement of gynecological organs. In contrast with the ovarian injury related to the type of antiblastic treatments, conditioning regimens and age (1,2,5), severe but not necessarily extensive cGVHD was the factor most closely related to the vaginal and cervical stenosis in our patients. Furthermore, permanently undetectable endometrium was observed in another woman, despite sequential EPT administration.

Although vulvo-vaginal infection had been described as causing vaginal and cervical stenosis, the post-transplant period was free of infection in the above described patients.
Holm *et al.(*6) has found a severe impact of total body irradiation (TBI) in association with SCT on the internal female genitalia, causing a permanent reduction in uterine and ovarian volumes, even during either EPT administration or spontaneous pubertal development. Similar findings have been reported by Bath *et al.* (7) who has shown reduced blood flow and undetectable endometrium in adult women who underwent SCT during childhood. Contrarily to Holm, the study by Bath found that physiological EPT improved both uterine blood flow and endometrial thickness. Similarly, reduced vaginal elasticity and rugal folds, pale tissue, atrophic vulvovaginitis, introital stenosis, and loss of pubic hair were noted by Schubert *et al*. (2) as frequent finding after allo-SCT, being more frequent in women who had previously been treated by TBI (33/36 *vs*. 2/8 of not treated by TBI). However, none of the women in our population had undergone TBI or other radiation treatment.

Two large studies on the reproductive outcome of SCT recipients were carried out (one in Europe, the other in the USA), both showing a high incidence of miscarriage, pre-term labor and low birth weight babies in female recipients of allografting (8,9). On the other hand, congenital malformation, developmental delay and malignant disease were similar in the offspring of SCT recipients compared to the general population. This suggests that mechanical problems or infections can cause the difference in the reproductive outcome between the allo-SCT setting and the general population.

Interestingly, gynecological manifestation of cGVHD described in the literature were always associated with the extensive disease. One of our patients was affected by the limited cGVHD with only severe liver involvement. Chronic GVHD represents a complex disorder characterized by reaction of immunocompetent donor lymphocytes against histocompatibility antigens of the host. Cutaneous changes have been described as virtually always present (10). Immunologically, pathologically and clinically, cGVHD is similar to autoimmune diseases, sharing features with lichen planus, scleroderma and Sjögren syndrome. Although no clinically apparent cutaneous lesions were detected in case no. 3, we cannot exclude that she had some mild occult cutaneous involvement.

Table 1. Characteristics of the women with gynecological manifestation of cGVHD

	Patient n. 1	**Patient n. 2**	**Patient n. 3**
Diagnosis	AML	CML	CML
Age at transplant (years)	30	24	21
Acute GVHD (grade)	0	1	1-2
Chronic GVHD	extensive	extensive	limited
sites involved	Muco-cutaneous, ophthalmic	Cutaneous, liver, ophthalmic	Liver
Introduction of EPT (mos. after transplant)	18	36	26
Glucocorticoid cumulative dose (g)	9.6	0.8	10
duration of treatment (days)	212	30	360
Cyclosporine A cumulative dose (g)	37.5	70	70.9
duration of treatment (days)	135	180	750

Abbreviations: GVHD; graft-versus-host disease; AML: acute myeloid leukemia, CML: chronic myeloid leukemia; HRT: hormone replacement therapy. Glucocorticoid dose is expressed as prednisone equivalents.

Progression or recurrence of lesions were previously observed in cases of extensive cGVHD, despite ongoing treatments (11). In our patients nos. 2 and 3, with extensive and limited cGVHD, respectively, who were sexually inactive, the withdrawal of local therapy with equine conjugated estrogens caused exacerbation of the vaginal lesions, even in the absence of any other sign of active GVHD. No systemic immunosuppressive treatment was given to our women because they were lacking any other manifestation of cGVHD, and vaginal dilatation with local application of estrogen improved consistently their condition. As a matter of fact, estrogen deficiency can contribute to the gynecological manifestation in SCT recipients, by worsening the atrophy of external and internal genitalia.

Continuous regimen of EPT might be less risky for hematocolpometra development. An important issue is represented by the psychological problems related to the lack of menstrual cycles, which is perceived as a loss of femininity, further worsening the patient's self-perception already damaged by the disease history and likelihood of infertility. Our young women preferred sequential treatment with periodical bleeding that made them feel not so different from "normal women". Pre-transplant counseling to female patients should also include information on their possible gynecological complications and outcomes.

Conclusion

In conclusion, gynecological complications in allo-SCT recipients are likely multifactorial, including contribution of TBI, cGVHD, estrogen deficiency and infections. Women undergoing allo-SCT are mostly young and have more that 90% probability of ovarian failure with very little possibility of menstrual cycles recovery. Findings of previous studies and our experience indicate that, following SCT, both uterine and ovarian functional status need to be investigated before choosing the appropriate management for any single women. All women with cGVHD should undergo gynecological examination before introducing EPT, in order to avoid unpleasant complication as hematocolpometra. Vaginal and cervical synecchiae can be treated with prolonged local estrogen treatments; temporary use of continuous EPT regimens might be preferable in these women. Moreover, patients with cGVHD should be closely monitored by pelvic exam and

ultrasonography during the first months of EPT, to detect any complication caused by intrauterine adhesions undetected at previous gynecological examinations.

References

1. Brennan, B & Shalet, SM. Endocrine late effects after bone marrow transplant. [Review] *Br J Haematol* 2002;118:58-66.
2. Schubert, MA, Sullivan, KM, Schubert, MM, *et al*. Gynecological abnormalities following allogeneic bone marrow transplantation. *Bone Marrow Transplant* 1990;5:425-430.
3. Delord C, Treleaven J, Shepherd J, Saso R, Powlers RL. Vaginal stenosis following allogeneic bone marrow transplantation for acute myeloid leukemia. *Bone Marrow Transplant* 1999; 23:225-52.
4. Corson, SL, Sullivan, K, Batzer, F, August, C, Storb, R, Thomas, ED. Gynecologic manifestation of chronic graft-versus-host disease. *Obstet Gynecol* 1982;60:488-492.
5. Tauchmanovà, L., Selleri, C., De Rosa, G., et al. Gonadal status in reproductive aged women after stem cell transplantation for haematological malignancies. *Hum Reprod* 2003; 18:1410-1416.
6. Holm, K, Nysom, K, Brocks, V, Hertz, H, Jacobsoen, N, Muller, J. Ultrasound B-mode changes in the uterus and ovaries and Doppler changes in the uterus after total body irradiation and allogeneic bone marrow transplantation in childhood. *Bone Marrow Transplant* 1999;23:259-263.
7. Bath, LE, Critchley, HOD, Chambers, SE, Anderson, RA, Kelnar, CJH, Wallace, WHB. Ovarian and uterine characteristics after total body irradiation in childhood and adolescence: response to sex steroid replacement. *British J Obstet Gynaecol* 1999;106:1265-1272.
8. Salooja, N, Szydlo, RM, Sociè, G, *et al.* Pregnancy outcomes after peripheral blood or bone marrow transplantation: a retrospective survey. *Lancet* 2001;358: 271-276.
9. Sanders, JE, Hawley, J, Levy, W, *et al.* Pregnancies following high-dose cyclophosphamide with or without high-dose busulphan or total-body irradiation and bone marrow transplantation. *Blood* 1996;87:3045-3052.
10. Cordoba, S, Vargas, E, Fraga, J, *et al.* Lichen sclerosus et atrophicus in sclerodermatous chronic graft-versus host disease. *Int J Dermatol* 1999;3:700-711.
11. Anguenot, JL, Ibèchèole, V, Helg, C, Piacenza, JM, Dumps, P, Bonnefoi, H. Vaginal stenosis with hematocolpometra, complicating chronic graft versus host disease. Case report. *Eur J Obstet & Gynecol Reprod Biol* 2002;103:185-187.

High serum leptin in patients with chronic graft versus host disease after hematopoietic stem cell transplantation

Introduction

Leptin, a protein encoded by the ob gene, is an adipocyte-derived hormone of the long-chain helical cytokine family playing a regulatory role in the neuronal control of body weight by inhibiting food intake and stimulating energy expenditure (1). The leptin receptor is expressed not only in the central nervous system, but also in several peripheral tissues, such as hematopoietic and immune systems (2,3). It is now becoming increasingly clear that leptin has important physiological functions in hematopoiesis, reproduction, immunity and inflammation (2-5). Clinical and experimental evidence also indicates that alterations in leptin levels and/or in the responsiveness to leptin may be involved in the development of several immune-related diseases such as diabetes, thyroiditis, rheumatoid arthritis, immune-mediated renal disease and multiple sclerosis (6-10). Leptin production has been shown to be sustained by several inflammatory cytokines both in rodents and in humans (11,12). Recently, it has been also documented that leptin exerts a direct effect on T-lymphocyte responses, promoting T-helper 1 (Th1) and suppressing Th2 cytokine production. In particular, leptin stimulates naive T-cell proliferation, enhances interferon-γ (IFN-γ and tumor necrosis factor-alpha (TNF-α production and decreases interleukin (IL)-4 secretion of memory T cells (10,13-15). Increased serum leptin values have been found after heart, liver, kidney and stem cell transplantation (SCT) (16-18). However, the reasons and the mechanisms responsible for such a post-transplant increase are still unclear. In the SCT setting, only a single paper has appeared, dealing with serum leptin levels in relation with conditioning protocols and growth hormone (GH) status (18). However, alterations in nutrition, endocrine and immune system functions occurring in SCT recipients might also influence leptin production. Changes in nutritional status can occur during the early post-transplant period due to a decrease in food intake and impaired gastrointestinal system function. The nutritional status can be further impaired after SCT due to treatments for acute or chronic graft versus host disease (aGVHD, cGVHD). A prolonged immune deficiency usually occurs, due to delayed and incomplete recovery of the immune system in both the autologous (auto-) and allogeneic (allo-) settings, which is negatively influenced by long-lasting treatments with immunosuppressive drugs in

allotransplanted patients (19,20). Indeed, glucocorticoids, the most often used immunosuppressive therapy, increase leptin production both *in vitro* and *in vivo* (21,22). Transient or permanent gonadal failure consequent to SCT may further increase leptin secretion (23).

To better understand changes in leptin production in subjects undergoing SCT, we measured serum leptin concentration in a group of 60 transplanted patients and in 60 healthy subjects with similar age and body mass index (BMI). Moreover, 15 patients undergoing allo-SCT were prospectively evaluated, with leptin determination prior to and after transplant. Relationship with age, gender, BMI, gonadal status, lymphocyte subpopulations, cytokine secretion, inflammation and time elapsed since transplant was investigated. Based on the evidence that leptin increase may contribute to the onset of immune response, we also investigated *in vitro* consequences of leptin blockade on T cell activation in a mixed lymphocyte reaction (MLR).

Materials & Methods

Patients

Sixty consecutive patients (36 allo-SCT and 24 auto-SCT, median age 35.4 years, range 18-45 years) were enrolled in this study. All patients had received SCT for a hematological malignancy in our institution; they were enrolled in this study between 12 and 72 (median, 26) months after SCT. All patients were disease-free at time of evaluation, in complete hematological remission. Patients in both transplant settings had similar age and BMI. BMI was calculated in all subjects as weight divided by height square (kg/m^2). No patient had received radiation as part of therapy before transplant. Patients' profile at study entry is summarized in Table 1. Patients allo- or auto-transplanted for acute or chronic myeloid leukemia or acute lymphoblastic leukemia received the same conditioning regimen BUCY2, including busulphan (16 mg/kg in 4 days) and cyclophosphamide (120 mg/kg in 2 days); patients with lymphomas or chronic lymphocytic leukemia were conditioned with the BEAM protocol, consisting of carmustine (300 mg/m^2 in 1 day), etoposide (200 mg/m^2 in 4 days), cytarabine (400 mg/m^2 in 4 days) and melphalan (140 mg/m^2 in 1 day). Allo-SCT recipients received unmanipulated bone marrow cells from HLA-identical siblings, whereas auto-SCT recipients received mobilized peripheral blood stem cells. In the allogeneic setting, 21 patients were affected by cGVHD: 5 limited and 13 extensive skin cGVHD associated with liver, gastrointestinal and eye localization in 10, 9 and 8 patients, respectively; the other patients had isolated liver (n=2) and lung (n=1) localization. Liver manifestation included a reversible

increase in liver enzymes (ALT: 50-1000 U/L; AST: 81-760 U/L and □-glutamyltransferase: 90-360 U/L) and cholestasis with alkaline phosphatase (450-790 U/L) and bilirubin (5-9.5 mg/dl) increase in all subjects that was associated with transient icterus in three patients. Skin lesions included lichen planus and focal epidermal atrophy. Ocular dryness (Sjogren-like syndrome) was the major ophthalmological manifestation, while oral mucosa dryness and lichenoid lesions were the upper gastrointestinal tract symptoms. Diarrhea associated with malabsorption were present in five patients. The clinical diagnosis of cGVHD was confirmed, if indicated, by histopathology of skin, liver or mucous membranes. All these patients had been treated by prednisolone at doses of 1 mg/kg, associated with CsA at doses ranging from 1 to 8 mg/kg/day for a period ranging 6-24 months (**Table 1**). No patient was on corticosteroid treatment at study entry, the drug having been withdrawn since at least three months. In addition, no patients showed intestinal cGVHD and significant weight loss. The control group consisted of 60 healthy individuals, including 15 donors of stem cells and 45 healthy subjects matched for age, sex and BMI. Informed consent was obtained by all subjects and the study was designed in accordance with the Helsinki II Declaration.

Table 1. Clinical caracteristics of autologous (auto-SCT) and alloge-neic (allo-SCT) transplanted patients

	Auto-SCT (n=36)	Allo-SCT (n=24)
Age: years	33 ±12	34±11
(range)	(16-48)	(18-45)
Gender: female/male	17/19	11/13
Diagnosis:		
Acute Myeloid Leukemia	16	12
Acute Lymphoblastic Leukemia	4	0
Chronic Myeloid Leukemia	14	0
Hodgkin's disease	2	9
Non Hodgkin's Lymphoma	0	3
Conditioning regimen:		
BU-CY2	36	12
BEAM	0	12

Leptin, hormonal and biochemical evaluation

Blood samples were obtained from all subjects between 08:00 and 10:00 a. m. after 8-10 h fasting. All samples were stored at –80° C until analysis. Measurements for serum leptin, C-reactive protein (CPR), FSH, LH, testosterone/17β-estradiol and cortisol were determined in the same blood sample by commercially available kits. Leptin was measured using RIA (Linco Research, St Louis, MO, USA); the sensitivity was 0.5 ng/ml and was expressed both as absolute values and as leptin/BMI ratio. Cortisol, testosterone and estradiol were measured using Immulite, solid phase chemoluminescent enzyme immunoassay (DPC, Los Angeles, CA, USA); FSH and LH with RIA Biodata S,p.A. (Rimini, Italy). ACTH was tested by double-antibody ^{125}I radioimmunoassay (DPC Los Angeles, CA, USA). Intra-assay and inter-assay coefficients of variation were below 7% and 15%, respectively, for all endocrine determinations. Gonadal insufficiency was considered in men when testosterone levels were below the normal range, and in amenorrhoic women when estradiol concentrations were lower and FSH higher than the normal range for reproductive aged women.

Measurement of serum Th1 and Th2 cytokines

Serum levels of IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ were measured on the same blood sample used for leptin and hormonal evaluation by a cytometric bead array kit (CBA kit, Becton-Dickinson, San Diego, CA, USA). Briefly, 50 μl of sample or standard reagent was mixed with 50 μl each of mouse anti-human cytokine-captured beads and specific phycoerythrin-conjugated detection antibody reagent. The mixture was incubated subsequently for 3 h at room temperature prior to washing and data acquisition using flow cytometry (FACScan, Becton-Dickinson). Data were acquired and analyzed using Becton-Dickinson, CBA software, according to the manufacturer's instructions.

CD4-to-CD8 ratio was analysed, using a FITC-conjugated murine anti-human CD4 combined with a PE-conjugated anti-human CD8 (both from Becton Dickinson), in uniformly set lymphocyte gates that usually contained >95% of lymphocytes. Proper isotypic controls were used in all assays. At least ten thousand events were acquired in the live cell gates for analysis.

Mixed lymphocyte reaction(MLR)

Isolated peripheral blood mononuclear cells (PBMCs) were obtained from 5 SCT recipients (stimulator cells) and their corresponding unrelated HLA-mismatched donors

(responder cells) through density gradient centrifugation using Ficoll. Responder PBMNCs were monocyte-depleted (PBL) by plastic adherence for 2 h at 37°C in 100% humidity and 5% CO_2. Stimulator cells were irradiated (30 Gy) before being cultured with responder cells. Responder and stimulator cells were cultured for 5 days in RPMI 1640, supplemented with 10% fetal calf serum (FCS), antibiotics, and 5×10^{-5} β_2-mercaptoethanol, at a concentration of 5×10^6 cells/mL and a ratio of 1:1, in a final volume of 200 µL/well, in flat-bottomed 96-well microculture plates. MLR was performed in absence and in presence of purified polyclonal rabbit anti-human leptin (anti-hOb, 10 µg/ml; kindly provided by Dr. Radek Sokol, BioVendor Laboratory Medicine, Inc., Brno, Czech Republic); as control affinity purified rabbit polyclonal IgG was utilized. T-cell proliferation was measured on day 6 after a 18-hour pulse with ^{3}H-thymidine (^{3}H-TdR) (0.037 MBq/well [1 µCi/well], specific activity 24.79×10^{-10} Bq/mmol [6.7 Ci/mmol]) (Amersham, Buckingham, United Kingdom). Thereafter, the cells were harvested on glass filter paper, and the counts per minute (cpm) were determined in a liquid scintillation counter (Betaplate, Wallac, Boston, MA). Results are expressed as the mean of triplicate cultures for each experiment. Ficoll, RPMI 1540, FCS, antibiotics and β_2-mercaptoethanol were purchased from Life Technologies, Gaithesburg, MD,USA.

Statistical analysis

Data are expressed as mean ± SD throughout text and tables. Data were analyzed by the two-tailed Student t test. The Mann-Whitney U test was used to compare the differences between groups. To assess the relationship between leptin, cytokines, CRP, BMI, age and time since transplant the Pearson correlation coefficient was calculated. Two-sided *P* values <0.05 were taken as significant.

Results

Serum leptin, biochemical and hormonal changes after SCT

We compared serum leptin concentrations between 36 long term survivors after allo-SCT, 24 survivors after auto-SCT and 60 age and BMI matched controls. Serum leptin was significantly higher in the patients compared to controls; in addition, it was higher in patients after allo- than after auto-SCT, expressed as absolute value and after normalization for BMI (**Table 2, Figure 1A**).

Table 2. Biochemical features in patients after allo- or auto-SCT, and in healthy controls

	Allo-SCT (n= 36)	**Auto-SCT** (n= 24)	**Controls** (n=60)
Age at evaluation (years)	34.9±11	36.1±12	35.5±2.4
Female/Male	17/19	11/13	28/32
Time from transplant (months)	28.9±15	23±16	-
BMI (kg/m^2)	25.99±4.7	25.6±3.45	25.2±0.8
Leptin (ng/ml)			-
Males	20.98 ± 13.3 *[a]	11.1 ± 8.35[b]	3.8 ± 1.2
Females	34.1 ± 12.6 *[a]	26 .53± 10.9 [b]	10 ± 2.5
Leptin/BMI			
Males	0.78 ± 0.54*[a]	0.475 ± 0.3 [b]	0.135 ± 0.05
Females	1.36 ± 0.7* [a]	1.03 ± 0.4 [b]	0.68 ± 0.1
Patients with AML#			
Leptin	23.6 ± 18	15.4 ± 14.5	
Leptin/BMI	0.97 ±0.8	0.53 ± 0.42	
Cortisol (ng/dl)	151 ± 63	159 ± 52	160 ± 17
C-reactive protein (mg/L)	0.6± 0.3 [b]	0.7 ± 0.4 [b]	0.35 ± 0.2

Values are expressed as mean ± SD. Abbreviations: BMI: body mass index. #: 16 patients in the allo-SCT and 12 in the auto-SCT group were affected by AML. Statistical analysis, *: p<0.05 *vs*. auto-SCT patients; [a]: p<0.001 *vs*. controls; [b]: <0.005 *vs*. controls.

In 20 patients who were prospectively evaluated prior and after allo-SCT, pre-transplant levels overlapped those of controls, while after SCT serum leptin increased two-to ten fold (**Figure 1B**). When gender was introduced into the analysis, post-transplant leptin and leptin/BMI ratio displayed a better separation between the allo- and auto-SCT groups (**Figure 1A**). The typical sexual dimorphism (higher leptin in females) persisted in both transplant settings but was set up at higher levels (**Table 2, Figure 1 A**).

Significantly higher leptin values were found in 19 patients affected by cGVHD than in those free of this complication, also when men and women were considered separately (**Table 3**). The difference remained significant even when normalized for differences in BMI. BMI was similar

in patients with and without cGVHD and did not change significantly after SCT compared to pre-transplant levels (data not shown).

Table 3. Serum leptin in patients after allogeneic SCT with or without chronic GVHD

	Patients cGVHD+ (n=19)	Patients cGVHD- (n=17)	*P* value
All patients			
BMI (kg/m2)	25.6 ± 1.6	25.9 ± 1.7	NS
Leptin (ng/ml)	29 ± 15	19 ± 14	0.007
Leptin/BMI	1.14 ± 0.6	0.72 ± 0.5	0.006
Females			
Leptin (ng/ml)	35 ± 10	22 ± 9	0.012
Leptin/BMI	1.4 ± 0.6	0.8 ± 0.7	0.03
Males			
Leptin (ng/ml)	27.8 ±13	14.0 ± 10	0.027
Leptin/BMI	0.95 ±0.43	0.5 ± 0.4	0.03

The data are expressed as mean±SD. BMI: body mas index.

Therefore, the increase in serum leptin induced by SCT was not a function of BMI variations. As expected, a significant correlation was found between leptin and BMI in controls, donors, patients prior to transplant and those auto-transplanted (r=0.475–0.68; $p<0.05$ for all); by contrast, no correlation was revealed in patients after allo-SCT (r= 0.057; $p=0.835$) (**Fig. 2**).

Significantly higher leptin level was present in 12 hypogonadic women (leptin and leptin/BMI ratio: 35.8±6.7 ng/ml and 1.38±0.19, respectively) compared to those with regular menstrual cycles or on hormone replacement therapy (HRT) (leptin and leptin/BMI ratio: 20.4± 4.6 ng/ml and 0.95±0.18, with $p=0.003$ and $p=0.002$, respectively).

Although circulating C-reactive protein (CRP), an acute phase inflammatory reactant, was higher in patients than in controls (**Table 3**), their values did not differ between the allo- and auto-SCT settings and between patients affected or not by cGVHD (**Table 3**). No patient had clinical or laboratory evidence of acute or chronic infection at the time of evaluation. By linear regression, leptin levels did not correlate with cortisol and CRP values, time since transplant and age when the patients were considered altogether, as subgroups (allo-/auto-transplanted) or women and men separately.

Cytokines analysis and CD4-to-CD8 ratio after SCT

Since a dysregulation of the type-1/type-2 cytokine balance may frequently occur following auto- and allo-SCT, we evaluated IL-4, IL-5, IL-10, IL-2, IFN-γ and TNF-α in the

same serum sample of auto- and allo-SCT patients used for measurement of leptin levels, using microparticle-based flow cytometric technology (**Table 4**). Mean level of IL-4, IL-5, IL-10 and IL-2 in auto- and allo-SCT patients did not differ from the baseline levels observed in 15 healthy subjects; there was no difference in the levels of these cytokines also between patients with and without cGVHD. As for IFN-γ and TNF-α their concentrations in auto-SCT patients were not different from those in normal subjects. In allo-SCT patients we found higher levels of circulating IFN-γ compared to auto-SCT patients and normal controls, whereas TNF-α concentrations did not differ among the three subgroups. Within allo-SCT patients, mean IFN-γ and TNF-α levels were higher in patients with than in patients without cGVHD. In addition, relationship between concentrations of TNF-α or IFN-γ and serum leptin levels were evaluated in allo-SCT patients. Patients with higher circulating IFN-γ concentrations, but not those with higher TNF-α levels, showed increased serum leptin levels. Indeed, using as a cut-off the median value of serum IFN-γ concentration (15 pg/ml that means the the median value of the patients' cohort), there was a significant difference in serum leptin levels between the groups of patients with higher or lower serum IFN-γ levels (33 vs. 19 mcg/L, p=0.04). Moreover, a significant correlation between leptin and IFN-γ was found only when allotransplanted patients with IFN-γ values > 15 ng/ml were considered.

As documented by the concomitant analysis of CD4-to-CD8 ratio, a significantly lower ratio was found both in auto-SCT (mean ± SEM: 0.95±0.1) and allo-SCT (mean ± SEM: 0.7±0.1) patients compared with normal individuals (normal range: 1.1-2.4; $p<0.05$). A majority of allo-SCT patients with cGVHD showed a CD4-to-CD8 ratio (mean ± SEM: 0.59±0.06) significantly lower than patients without cGVHD (mean ± SEM: 0.97±0.1; p=0.001). However, the correlation between serum leptin levels and degree of decrease of CD4-to-CD8 ratio did not reach statistical significance (p=0.09).

Effects of leptin-blockade in the mixed lymphocyte reaction(MLR)

In order to elucidate the involvement of serum leptin in alloreactivity, we tested the influence of a polyclonal rabbit anti-human leptin antibody in a one-way MLR in which PBMNC from 5 unrelated HLA-mismatched donors were stimulated with PBL from their corresponding recipients. As shown in the **Figure 3**, there was a significant reduction in T-lymphocyte proliferation when mixed cultures of T lymphocytes stimulated by irradiated allogeneic PBMNCs (1:1 ratio) were performed in the presence of a leptin-blocking antibody (mean decrease of proliferative responses > 50%, range 40-60%, as measured by ^{3}HTdR incorporation; p = 0.001).

Table 4 - Mean serum cytokine levels in transplanted patients

	IL-2	IL-4	IL-5	IL-10	IFN-α	TNF-γ
Controls (n=15)	2.5 ± 0.4	3.8 ± 0.7	2.5 ± 0.6	3.4 ± 0.9	6.5 ± 1.2	3.0 ± 0.5
Auto-SCT (n=20)	1.9 ± 0.5	4.0 ± 1.0	2.1 ± 0.3	4.0 ± 1.1	8.8 ± 1.3	3.9 ± 1.1
Allo-SCT (n=35)	3.2 ± 0.2	5.0 ± 0.5	3.8 ± 0.6	6.7 ± 0.4	14.4 ± 1.1*°	4.7 ± 0.4
without cGVHD (n=14)	3.1 ± 0.2	4.4 ± 0.9	3.1 ± 0.9	4.0 ± 1.1	9.8 ± 1.5	3.5 ± 0.5
with cGVHD (n=21)	3.3 ± 0.3	5.3 ± 0.5	4.3 ± 0.9	7.4 ± 1.2	17.1 ± 1.3 [a]	5.5 ± 0.3 [b]

Values are expressed as mean pg/ml ± SEM. Statistical analysis, *: p=0.0001 vs. controls; °: p=0.01 vs. auto-SCT patients; [a]: p=0.001 *vs.* patients with cGVHD ; [b]: p =0.04 *vs.* patients with cGVHD.

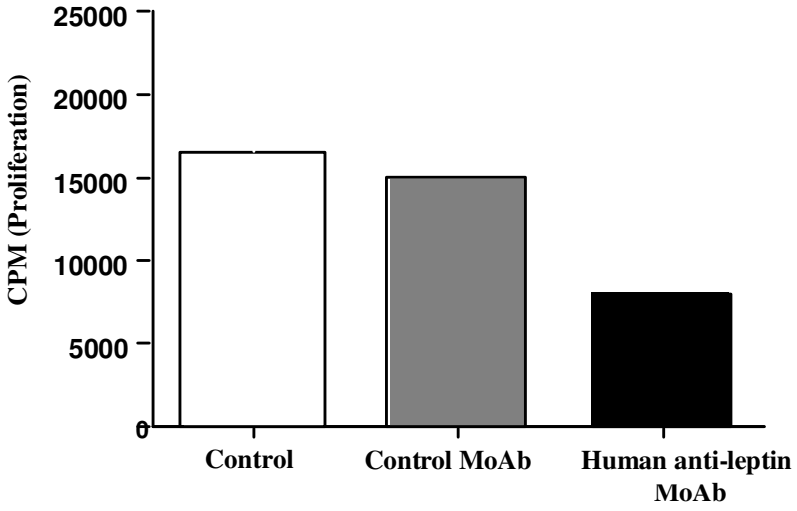


Figure 3.

Figure 3. Effect of leptin-blockade on T cell proliferation in a representative allogeneic mixed lymphocyte reaction (MLR). Freshly isolated peripheral blood lymphocytes (PBL) from a HLA-mismatched recipient were cultured at 1:1 ratio with irradiated peripheral blood mononuclear cells (PBMNCs) from the donor as stimulators, in medium alone, with purified rabbit anti-human leptin (anti-hOb) or with polyclonal rabbit anti-human IgG used as control (CTR-ab). Treatment with polyclonal anti-hOb induced 50% decreased (7981±1969) proliferation in response to HLA-mismatched PBMNC stimulators compared to medium alone (16141±5393) and CTR-ab (14795±2325). Columns and error bars represent mean cpm values and standard deviation of triplicate cultures.

Little, if any, inhibition occurred in the presence of a polyclonal rabbit anti-human IgG utilized as control in MLR (mean decrease of proliferative responses < 4%, range 0-8%; $p = 0.1$).

Discussion

In agreement with a previous report (18), we found that serum leptin levels were significantly increased after SCT in our cohort of patients. In prospectively evaluated patients, two-to-ten fold increase in serum leptin was observed after allo-SCT, pointing out that increased leptin production was consequent to the SCT procedure and not due to the basic disease. In the general population serum leptin is proportional to BMI; it is reduced by starvation and increased

by food intake (24,25). BMI is commonly used as an indirect index of fat mass in clinical practice. In our study, serum leptin correlated with BMI in controls and auto-transplanted patients, but not in allo-transplanted patients, suggesting that factors other than BMI can strongly influence leptin overproduction in this subgroup. Indeed, in these patients, leptin increase was not a function of the BMI changes, since BMI was similar in allo- and auto-transplanted patients as well as in allo-transplanted patients before and after transplant. The typical sexual dimorphism in leptin distribution was preserved in our patients' population, although set at a higher level.

In the previous study on leptin after SCT (18), the effects of radiation therapy on the hypothalamic-pituitary region and/or direct irradiation of the adipose tissue were hypothesized as possible causes of the leptin increase, and the negative relationship between growth hormone peak and leptin was explained by possible radiation-induced hypothalamo-pituitary lesions. Our data do not support such hypotheses. In fact, none of our patients had received radiation therapy as part of treatment for the underlying disease before transplant or as part of their conditioning regimen. In addition, in a recent study from our group on endocrine function in allo-SCT recipients, no evidence of hypothalamic-pituitary lesion capable of influencing leptin secretion was found (26). Leptin increase should not be caused by the underlying disease, as its levels were shown decreased in untreated patients affected by AML, without any significant change during chemotherapy-induced cytopenia and neutropenia (27). The differences in leptin levels in patients after auto- and allo-SCT were similar when the analysis included the whole group of patients or only patients with AML diagnosis.

Recently, leptin has been proposed to act as a link between nutritional status and cell-mediated immunity (11). BMI was similar in controls and in auto- and allo-transplanted patients, indicating that the difference in leptin values was not caused by the host's nutritional status. On the other hand, no patients showed signs of intestinal GVHD and alteration of the nutritional status.

Chronic GVHD remains a major cause of morbidity and mortality in allo-SCT (28). Significantly higher leptin levels were found in patients affected by cGVHD compared to those free of this complication and the difference was even more evident when men and women were considered separately. This induced us to investigate more accurately the relationship between cGVHD and serum leptin.

Chronic GVHD is associated with a state of profound immunodeficiency, which can easily lead to infections, particularly when prolonged immunosuppression is required for its treatment (29). However, no patient was on corticosteroid treatment or had adrenal insufficiency at time of testing, as documented by normal serum cortisol and plasma ACTH levels. Furthermore, the lack

of correlation between cortisol and leptin values suggests no significant influence of cortisol on serum leptin levels in our cohort. Stimulating effect of estrogens and progesterone on leptin production has previously been described (30,31), with leptin lowering after menopause (32). However, we found higher leptin levels in hypogonadal women compared to those with regular menstrual cycles or on HRT. In addition, all women with cGVHD suffered from ovarian failure and were not assuming HRT. Taken together, all these findings support the hypothesis that factors other than a dysregulation of the endocrine system may influence the increase of serum leptin levels in cGVHD patients after allo-SCT.

Increase in circulating leptin was found during fever and systemic inflammation (4,33). No our patients had clinical evidence of acute or chronic infection at time of evaluation. As serum CRP positively correlates with leptin increase during systemic inflammation, (34,35) we investigated this relationship in our patients. Lack of correlation between leptin and CRP further indicated that leptin increase in our cohort of patients was not caused by a systemic inflammatory state.

Accumulating evidence documents that leptin play a role in several autoimmune diseases (6-10). Chronic cGVHD is a particular type of immune-mediated process. It may be caused by an immunological attack of target organs by transplanted donor lymphocytes as extension of the alloreactivity responsible for acute GVHD, or it can be a manifestations of an altered immune reconstitution with generation of autoreactive T-cell clones and dysregulation of post-thymic $CD4^+$ effector memory cells (CCR7-/CD62low) and $CD4^+CD25^+$ cells (36,37). Indeed, cGVHD shares similarities with the mechanisms involved in several autoimmune disorders, namely collagen-vascular diseases (38,39). The up-regulation of the Th1 cytokines IFN-gamma and IL-12 in mononuclear cells of cGVHD patients implicates that also a Th1-driven mechanism may be involved in the development of this chronic complication (40,41). Furthermore, IFN-γ plays a pivotal role in the increased collagen deposition, a characteristic pathologic feature of cGVHD (42,43). In view of the higher serum leptin levels after SCT and of its peculiar increase after cGVHD, we investigated their relationship with the Th1 and Th2 cytokines secretion pattern and with the circulating CD4-to-CD8 ratio, which are frequently unbalanced following auto- and allo-SCT, especially when cGVHD is present. Among the cytokines secreted by type-1 and type-2 T-helper lymphocytes, serum IFN-γ and TNF-α levels were significantly higher in patients who developed GVHD compared with auto-transplanted patients and healthy controls. However, only serum IFN-γ levels correlated significantly with increased serum leptin in allo-SCT patients, in particular in those with increased IFN-gamma suggesting more close relationship when the immune system is activated.

Interactions between leptin and several cytokines have been previously reported. In particular, proinflammatory Th1 cytokines increase leptin secretion by adipose tissue (44); leptin in turn stimulates the production of Th1 type cytokines from stimulated lymphocytes (3,4,10,11,14,15,45). Furthermore, leptin regulates the balance of Th1/Th2 cytokines (13). Thus, it is possible that Th1 lymphocyte activation during cGVHD may account for the difference in leptin values observed between auto- and allo-transplanted subjects. A major increase in serum leptin levels in patients with cGVHD suggests that autoreactive T cells involved in cGVH reaction may further stimulate leptin secretion. In addition, as leptin reduces T cells apoptotic rate (45), one may guess that leptin allows escape of lymphocyte subpopulations with anti-host reactivity, thus worsening cGVHD. Minor increase in serum leptin levels and preserved leptin/BMI ratio observed after auto-SCT are in line with a milder immune system alteration in this cohort of patients, as documented by a less altered CD4-toCD8 ratio.

Although the hypothesis that high serum leptin levels represents an epiphenomenona of cGVHD has also to be taken into a consideration, our data suggest that leptin increase may have some role in the development and maintenance of cGVHD. Previous evidence exists that leptin addition to MLR increases T-lymphocyte proliferation and IFN-γ secretion (10-12). More recently, it has been shown that Th1 cells and macrophages secrete leptin that sustain their function through an autocrine loop (3,13). To investigate the involvement of immune-mediated abnormalities in the triggering of leptin secretion in cGVHD+ patients, we tested the role of leptin in the allogeneic MLR, an *in vitro* model of T-cell activation that takes place during allografting *in vivo*. Substantial decrease in proliferative responses by activated alloreactive T cells was achieved by incubating one-way MLR from 5 HLA-mismatched couples of responder and stimulator T-lymphocytes with a blocking anti-human leptin polyclonal antibody. These data suggest that 1) leptin can act as an autocrine growth factor in MLR and 2) serum leptin increase during cGVHD can also be ascribed to T cell-derived leptin, apart to be due to a well known IFN-γ and TNF-α–induced leptin production by adipose tissue.

In conclusion, we found greater increase in serum leptin after allo-SCT than auto-SCT, with the highest levels in patients with cGVHD. Loss of the physiological relationships between leptin, BMI and gonadal function, the correlation between serum leptin and IFN-γ levels in the allogeneic setting, and the marked inhibitory effect of leptin-blockade on T cell activation in primary MLR suggest that T-cell activation after allo-SCT may be involved in the leptin increase, and that the latter might subsequently contribute to the maintenance of the immune attack responsible for cGVHD.

References

1. Friedman JM. Halaas JL. Leptin and the regulation of body weight in mammals. Nature 1998; 395:763.
2. Konopleva M, Mikhail A, Estrov Z, et al. Expression and function of leptin receptor isoforms in myeloid leukemia and myelodysplastic syndromes: proliferative and anti-apoptotic activities. Blood. 1999;93:1668.
3. Matarese G, Sanna V, Fontana S, Zappacosta S. Leptin as a novel therapeutic target for immune intervention. Curr Drug Targets Inflamm Allergy. 2002;1:13.
4. Fantuzzi G, Faggioni R. Leptin in the regulation of immunity, inflammation, and hematopoiesis. J Leukoc Biol. 2000; 68:437.
5. Kratzsch J, Lammert A, Bottner A, et al. Circulating soluble leptin receptor and free leptin index during childhood, puberty, and adolescence. J Clin Endocrinol Metab. 2002;87:4587.
6. Moran O, Phillip M. Leptin: obesity, diabetes and other peripheral effects. Pediatr Diabetes. 2003;4:101.
7. Zimmermann-Belsing T, Brabant G, Holst JJ, Feldt-Rasmussen U. Circulating leptin and thyroid dysfunction. Eur J Endocrinol. 2003 Oct;149:257.
8. Bokarewa M, Bokarew D, Hultgren O, Tarkowski A. Leptin consumption in the inflamed joints of patients with rheumatoid arthritis. Ann Rheum Dis. 2003;62:952.
9. Schroth M, Kratzsch J, Groschl M, Rauh M, Rascher W, Dotsch J. Increased soluble leptin receptor in children with nephrotic syndrome. J Clin Endocrinol Metab. 2003;88:5497.
10. Sanna V, Di Giacomo A, La Cava A, et al. Leptin surge precedes onset of autoimmune encephalomyelitis and correlates with development of pathogenic T cell responses. J Clin Invest. 2003;111:241.
11. Faggioni R, Feingold KR, Grunfeld C. Leptin regulation of the immune response and the immunodeficiency of malnutrition. FASEB J. 2001;15:2565.
12. Bruun JM, Pedersen SB, Kristensen K, Richelsen B. Effects of pro-inflammatory cytokines and chemokines on leptin production in human adipose tissue in vitro. Mol Cell Endocrinol. 2002;190:91.
13. Lord GM, Matarese G, Howard JK, Baker RJ, Bloom SR, Lechler RI. Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. Nature. 1998;394:897-901.
14. Faggioni R, Jones-Carson J, Reed DA, et al**.** Leptin-deficient (ob/ob) mice are protected from T cell-mediated hepatotoxicity: role of tumor necrosis factor alpha and IL-18. Proc. Natl. Acad. Sci. USA. 2000;97:2367.
15. Matarese G, Di Giacomo A, Sanna V, et al. Requirement for leptin in induction and progression of experimental autoimmune encephalomyelitis. J. Immunol. 2001;166:5909.
16. Winnicki M, Phillips BG, Accurso V, et al. Independent association between plasma leptin levels and heart rate in heart transplant recipients. Circulation. 2001;104:384.
17. Kagan A, Haran N, Leshinsky L, et al. Serum concentrations of leptin in heart, liver and kidney transplant recipients. Isr Med Assoc J. 2001; 4:213.
18. Couto-Silvia AC, Trivin C, Esperou H, Michon J, Fischer A, Brauner R. Changes in height, weight and plasma leptin after bone marrow transplantation. Bone Marrow Transpl. 2000; 26,1205.
19. Imamura M, Tsutsumi Y, Miura Y, Toubai T, Tanaka J. Immune reconstitution and tolerance after allogeneic hematopoietic stem cell transplantation. Hematology. 2003;8:19.
20. Porrata LF, Litzow MR, Markovic SN. Immune reconstitution after autologous hematopoietic stem cell transplantation. Mayo Clin Proc. 2001;76:407.
21. Dagogo-Jack S, Umamaheswaran I, Askari H, Tykodi G. Leptin response to glucocorticoid occurs at physiological doses and is abolished by fasting. Obes Res. 2003;11:232.
22. Williams LB, Fawcett RL, Waechter AS, et al. Leptin production in adipocytes from morbidly obese subjects: stimulation by dexamethasone, inhibition with troglitazone, and influence of gender. J Clin Endocrinol Metab. 2000;85:2678.
23. Mantzoros CS. Role of leptin in reproduction. Ann N Y Acad Sci. 2000;900:174.

24. Gomez JM, Maravall FJ, Gomez N, Navarro MA, Casamitjana R, Soler J. Interactions between serum leptin, the insulin-like growth factor-I system, and sex, age, anthropometric and body composition variables in a healthy population randomly selected. Clin Endocrinol. 2003; 58:213.

25. Considine RV. Regulation of leptin production. Rev Endocr Metab Disord. 2001;2:357.

26. Tauchmanova L, Selleri C, De Rosa G, et al. High prevalence of endocrine dysfunction in long-term survivors after allogeneic bone marrow transplantation for hematologic diseases. Cancer. 2002;95:1076.

27. Bruserud O, Huang TS, Glejen N, Gjertsen BT, Foss B. Leptin in human acute myelogeneous leukaemia: studies of in vitro levels and in vitro effects on native functional leukaemia blasts. Haematologica. 2002; 87:562-563.

28. Lee SJ, Klein JP, Barrett AJ, et al. Severity of chronic graft-versus-host disease: association with treatment-related mortality and relapse. Blood. 2002;100:406.

29. Lee SJ, Vogelsang G, Flowers ME. Chronic graft-versus-host disease. Biol Blood Marrow Transplant. 2003;9:215.

30. Kitawaki J, Kusuki I, Koshiba H, Tsukamoto K, Honjo H. Leptin directly stimulates aromatase activity in human luteinized granulosa cells. Mol Hum Reprod. 1999;5:708.

31. Brann DW, De Sevilla L, Zamorano PL, Mahesh VB. Regulation of leptin gene expression and secretion by steroid hormones. Steroids. 1999;64:659.

32. Di Carlo C, Tommaselli GA, Pisano G, et al. Serum leptin levels in postmenopausal women: effects of transdermal hormone replacement therapy. Menopause. 2000;7:36.

33. Matarese G, La Cava A, Sanna V, et al. Balancing susceptibility to infection and autoimmunity: a role for leptin? Trends Immunol. 2002;23:182.

34. Maruna P, Gurlich R, Frasko R, Haluzik M. Serum leptin levels in septic men correlate well with C-reactive protein (CRP) and TNF-alpha but not with BMI. Physiol Res. 2001;50:589.

35. Sturmer T, Brenner H, Koenig W, Gunther K.P Severity and extent of osteoarthritis and low grade systemic inflammation as assessed by high sensitivity C reactive protein. Ann Rheum Dis. 2004;63:200.

36. Clark FJ, Gregg R, Piper K, et al. Chronic graft-versus-host disease is associated with increased numbers of peripheral blood CD4+CD25high regulatory T cells. Blood. 2003 Nov 6 [Epub ahead of print]

37. Xystrakis E, Bernard I, Dejean AS, Alsaati T, Druet P, Saoudi A. Alloreactive CD4 T lymphocytes responsible for acute and chronic graft-versus-host disease are contained within the CD45RChigh but not the CD45RClow subset. Eur J Immunol. 2004;34:408.

38. Ferrara JL. Cytokine dysregulation as a mechanism of graft-versus-host disease. Curr Opin Immunol 1993; 5: 794.

39. Fowler DH, Gress RE. Th2 and Tc2 cells in the regulation of GVHD, GVL, and graft rejection: considerations for the allogeneic transplantation therapy of leukemia and lymphoma. Leuk Lymphoma. 2000;38:221.

40. Korholz D, Banning U, Bonig H, et al. The role of interleukin-10 (IL-10) in IL-15-mediated T-cell responses. Blood. 1997;90:4513.

41. Tanaka J, Imamura M, Kasai M, et al. The important balance between cytokines derived from type 1 and type 2 helper T cells in the control of graft-versus-host disease. Bone Marrow Transplant. 1997;19:571-576

42. Teshima T, Ferrara JL. Understanding the alloresponse: new approaches to graft-versus-host disease prevention. Semin Hematol 2002; 39:15-22.

43. Parkman R. Chronic graft-versus-host disease. Curr Opin Hematol. 1998; 5:22-25.

44. Bruun JM, Pedersen SB, Kristensen K, Richelsen B. Effects of pro-inflammatory cytokines and chemokines on leptin production in human adipose tissue in vitro. Mol Cell Endocrinol. 2002; 190:91-99.

45. Sanchez-Margalet V, Martin-Romero C, Santos-Alvarez J, Goberna R, Najib S, Gonzalez-Yanes C. Role of leptin as an immunomodulator of blood mononuclear cells: mechanisms of action. Clin Exp Immunol. 2003;133:11.

46. Fujita Y, Murakami M, Ogawa Y, et al. Leptin inhibits stress-induced apoptosis of T lymphocytes. Clin Exp Immunol. 2002;128:21

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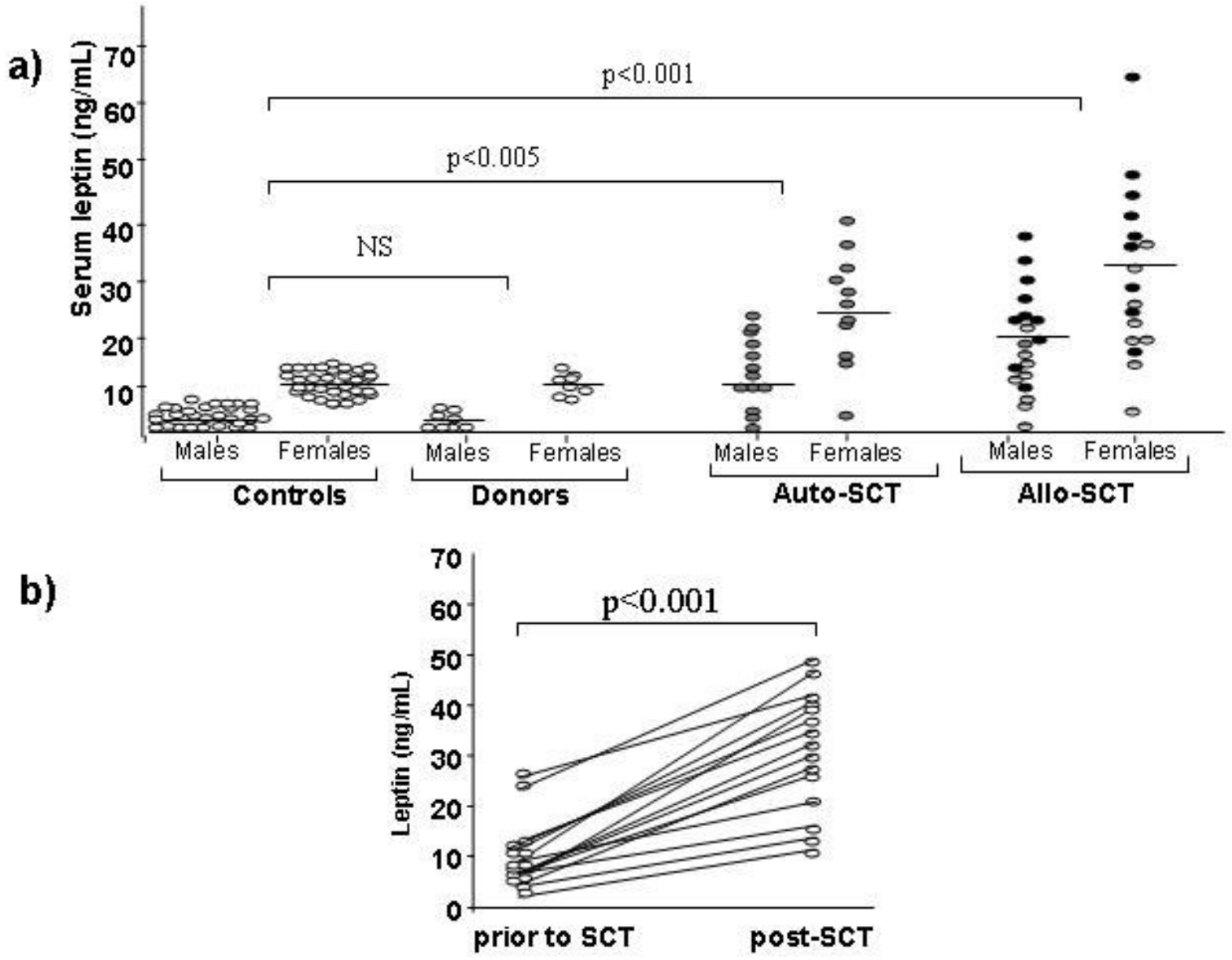

Figure 1. Serum leptin levels are significantly increased after autologous (auto) and allogeneic (allo) stem cell transplantation (SCT). Panel a: serum leptin levels in the various groups studied. Black dots: allo-SCT with chronic GVHD (cGVHD). Panel b: serum leptin modifications in the same patients before and after SCT.

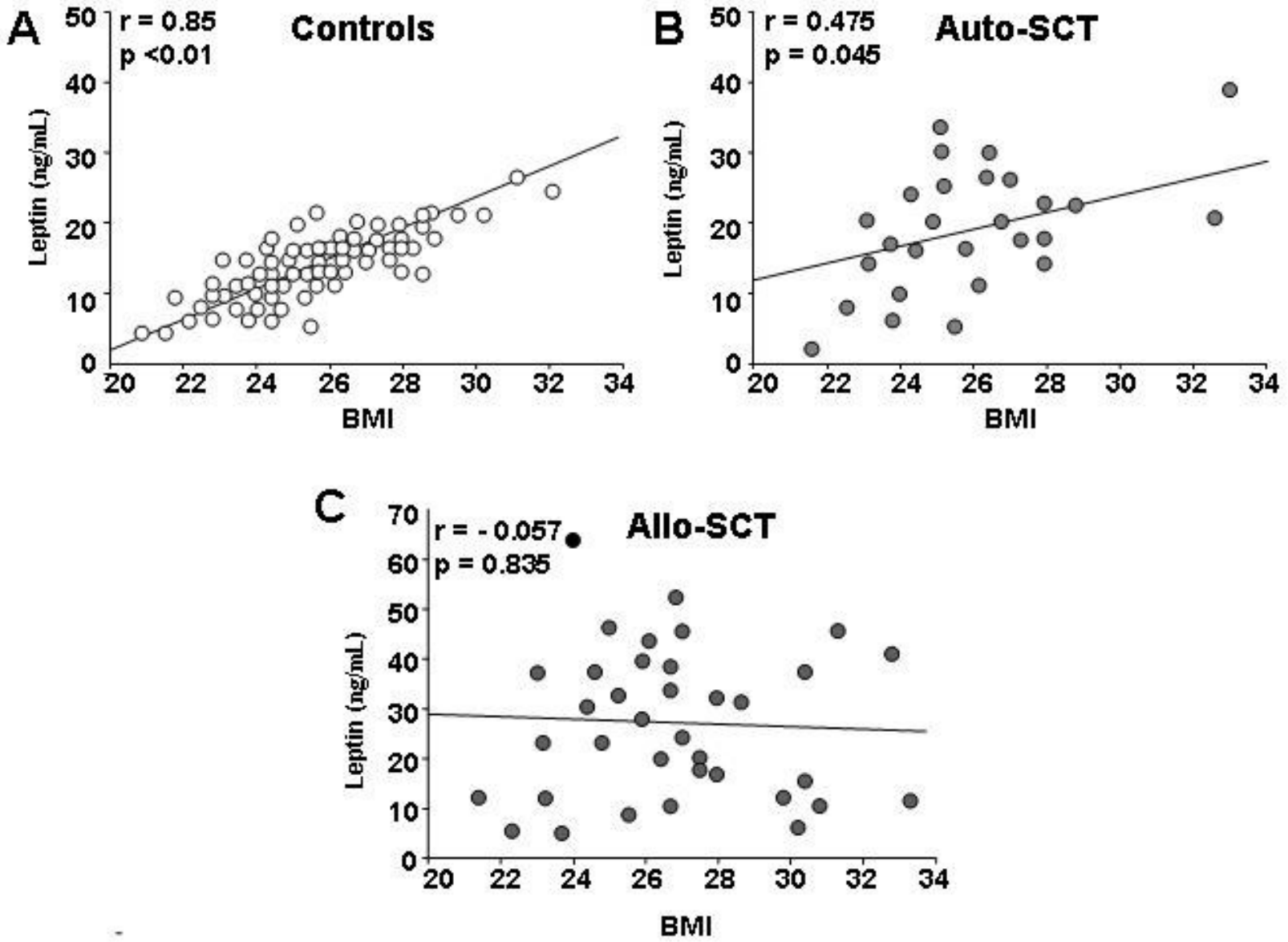

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Figure 2. Correlation between leptin and body mass index (BMI) is preserved in auto-SCT patients and lost in allo-SCT patients. Each dot represents a subject studied.

Long-lasting bone damage detected by dual energy x-ray absorptiometry, phalangeal osteosonogrammetry and *in vitro* growth of marrow stromal cells after allogeneic stem cell transplantation

Introduction

In the past few years the use of allogeneic stem cell transplant (allo-SCT) in the treatment of hematological diseases has grown exponentially along with a progressive improvement in patients' outcome due to a fall in transplant-related mortality (1). As the population of post-transplant long-term survivors is rapidly growing, attention is now focused on early and late complications of this procedure. Osteoporosis, fractures and avascular necrosis (AVN) are considered common complications leading to pain and disability, which negatively affect lifestyle and quality of life of transplanted patients (2,3). A significant decrease in bone mass has been shown in heart, kidney, lung and liver transplanted patients as well as in survivors after autologous and allo-SCT (4-12). The mechanisms of bone metabolism alteration caused by allo-SCT are multiple and not completely understood (13). Major risk factors for transplant-related osteoporosis include myeloablative conditioning regimens, cytokine storm at the time of transplantation, post-transplant long-lasting high-dose steroids and cyclosporin-A (CsA) therapy, immobilization and decreased kidney, liver and bowel function that result in reduced intake and altered metabolism of calcium and vitamin D (8,13-15). In addition, most women experience ovarian failure after allo-SCT (16).

Osteoporosis is defined as compromised bone strength and increased susceptibility to fractures, while osteopenia represents a less severe bone abnormality (17). Altered biochemical markers of bone metabolism have been found early after allo-SCT, suggesting a transplant-induced increase in bone turnover (18-20). In line with this finding, a high prevalence of osteoporosis has been found at trabeculae-rich sites within 12 months after allo-SCT (9,11,21). Dual energy X-ray absorptiometry (DEXA) is currently the best well-referenced method to diagnose transplant-related bone loss; quantitative computed tomography has also been adopted in one study (10). Although BMD, a parameter determined by DEXA and tomography, accounts for about 70% of bone strength, there is increasing evidence that other structural aspects that largely contribute to fracture risk are not detected by these techniques (23-25). Multiple risk factors related to SCT influence both mineral content and cellular components of bone, which cause modification of bone

microarchitecture and mechanical properties (13). Phalangeal OSG, as detected by quantitative ultrasonometry, is a recent non-invasive and radiation-free method that provides information on bone density and mechanical properties of bone including density, elasticity and width of trabeculae by assessing amplitude and speed of ultrasound signals crossing the bone (26-29). Phalanges show an equal proportion of compact and cancellous bone as well as a high metabolic turnover, which make them an important skeletal site for early identification of bone changes (30).

Bone is constantly regenerated by a process of osteoclast-mediated resorption and osteoblast-induced replacement; alterations of this equilibrium are the primary causes of osteoporosis. Osteoclasts are multinucleated hemopoietic cells derived from the monocyte/macrophage lineage, whereas osteoblasts are mesenchymal derived marrow stromal cells which allow the formation and mineralization of secreted bone matrix (31). Estimation of bone remodeling includes quantification of marrow colony forming units-fibroblast (CFU-F) cells *in vitro*. Currently, CFU-F are believed to be the best "*in vitro"* surrogate for the most primitive precursors for osteoblasts (32). Chemotherapy used for hematological malignancies may damage the function of microenvironment precursors, whereas myeloablative conditioning regimens followed by autologous or allo-SCT seem to delay regeneration of bone microenvironment (33,34).

The main aim of this cross-sectional study was to assess bone damage in adults with functioning donor marrow graft lasting more than one year. In particular, we focused on bone densitometry perfomed one to ten years after allo-SCT at three skeletal sites by two different methods: DEXA and OSG. Finally, densitometric results were compared to *in vitro* recovery of clonogenic fibroblast progenitors, which represents a pivotal step of bone remodeling.

Subjects and Methods

Patients and transplantation procedures

We evaluated bone abnormalities in forty-one consecutive patients who had been successfully allotransplanted at least one year before entering the study, with unmanipulated marrow from a HLA identical sibling. Reasons for transplant included patients with acute myeloid leukemia (n=18), chronic myeloid leukemia (n=17), acute lymphoblastic leukemia (n=4) and Hodgkin's lymphoma (n=2). There were 20 women and 21 men, with a median age of 28.5 yr (range, 14 –50) at SCT, and 32 yr (range, 20-51) at time of bone evaluation. All patients had been conditioned with the BU-CY2 regimen (busulphan 16 mg/kg and cyclophosphamide 120 mg/kg). All patients had also received CsA (1 mg/kg/day by continuous i.v. infusion from day -1 to day +

20 and then 8 mg/kg/day orally) plus short-course methotrexate as prophylaxis for graft versus host disease (GVHD). No patient had received prophylactic therapy with growth factors after SCT. Twenty-three patients developed acute GVHD (aGVHD) of global grade I to IV and were successfully treated with methylprednisolone at doses ranging from 2 to 10 mg/kg, then tapered as tolerated. Twenty-nine patients had been or were affected by chronic GVHD (cGVHD) (10 limited and 19 extensive form), treated with prednisolone at doses of 1-2 mg/kg, associated with CsA at doses ranging from 1 to 8 mg/kg/day.

Table 1. Clinical characteristics of patients who underwent allogeneic stem cell transplant.

Patient characteristics	**n=41**
Age at SCT (yrs)	31.4±11
Gender (F/M)	20/21
Underlying disease	
AML in 1st CR	18
CML in chronic phase	17
ALL in 2nd CR	4
HD	2
Conditioning regimen:	
BU-CY2	41
Age at evaluation (yrs)	34.9±9.8
BMI (kg/m^2)	23.9±5.4
Amenorrhea duration (months)	23.1±22.0
Acute GVHD	23
Grade I-II/III-IV	20/3
Chronic GVHD	29
Limited/extensive form	10/19
Corticosteroid treatment	
Duration (days)	260.8±245.0
Cumulative dose (g)*	6.7±5.5
Cyclosporine A treatment	
Duration (days)	340.4 ±194.8
Cumulative dose (g)	66.9±46.88

Values are expressed as mean ± SD. Abbreviations: SCT: stem cell transplant, AML: acute myeloid leukemia, CML: chronic myeloid leukemia, ALL: acute lymphoblastic leukemia, HD: Hodgkin's disease. CR: complete remission, BU: busuphan, CY: cyclophosphamide, BMI: body mass index; GVHD: graft versus host disease; Symbols: *: expressed as prednisone equivalents.

Calcium intake was estimated by description of the usual diet and ranged from 500 to 800 mg/day; daily supplemental calcium intake recommended for patients receiving steroid therapy was 1000 mg. Body mass index (BMI) was calculated for each patient as weight/height square (kg/m^2). No patient smoked, drank alcohol or more than 3 cups of coffee per day. The clinical characteristics of the patients are summarized in **Table 1**. The control group consisted of 188 healthy individuals, including family members and donors of stem cells, matched for age, sex and BMI. Informed consent was obtained from all patients and controls in accordance with institutional guidelines and the study design was made in accordance with the Helsinki II Declaration.

The median cumulative dose of steroids given to this cohort of patients was equivalent to 5.8 g of prednisone (range 0.8 - 24) for a period ranging from 3 to 36 months.

Hormonal and biochemical evaluation

Blood samples were obtained from all patients between 08:00 and 10:30 a.m every 3-6 months after SCT. Serum FSH, LH, 17-β-estradiol and testosterone were monitored to assess gonadal function; measurements were performed by commercially available kits: FSH and LH with radioimmunoassay (RIA, Biodata, Rimini, Italy), testosterone and estradiol using solid phase chemoluminescent enzyme immunoassay (DPC, Los Angeles, CA, USA). At study enter, serum calcium (Ca), phosphorus (P), creatinine, alkaline phosphatase (ALP), albumin, intact molecule parathyroid hormone (iPTH) and osteocalcin were determined after at least 8 hour fasting, while urinary calcium and hydroxyproline excretion were measured in 24h- urine collection, and corrected for creatinine excretion. Intact PTH and serum osteocalcin levels were measured by RIA (Nichols Institute Diagnostics, CA); the detection limit of the latter was 0.35 mg/L. Hydroxyproline excretion was measured by high pressure liquid chromatography. Blood chemistry profile, including levels of Ca, P, ALP, 24-h urinary Ca excretion and creatinine, were analyzed using a standard autoanalyzer.

Bone density evaluation, fracture and avascular necrosis assessment

Bone density was determined simultaneously at three different skeletal sites by two methods: DEXA and phalangeal OSG. Lumbar spine (LS) (L1-L4) and femoral neck (FN) BMD were measured by DEXA, using the Hologic QDR 1000 densitometer (Hologic, Inc., Waltham, MA). Individual BMD values are expressed as g/cm^2, T- and Z-scores. Quality control was maintained by daily scanning of an anthropomorphic spine phantom. The coefficient of variation for the DEXA technique was < 1% for the lumbar spine and 1.5% for the FN. The reference population adopted in this study was the international pooled sample provided by the manufacturer; their data, however, did not differ significantly from those obtained on a local sample in a study performed when the device was set up (35).

Phalangeal OSG evaluation was performed using DBM Sonic Bone Profiler (Igea, Carpi, Mo, Italy) as previously described (36). The amplitude-dependent speed of sound (AD-SoS) and ultrasound bone profile index (UBPI) were measured (27). Measurements were carried out on the 2^{nd} to the 5^{th} proximal phalanges of the non-dominant hand and the device automatically averaged the AD-SoS values of the four fingers. AD-SoS results were reported as m/s and as T- and Z-scores. The latter two parameters were calculated with the software provided by the manufacturer using

measurements obtained in a sample of Italian population as reference database. UBPI is an optimum mathematical combination of three signal parameters developed in order to better discriminate fracture risk (27) . It represents the probability of a single subject to belong to the nonfractured group; its values are normalized and range from 0 to 1, 1 being attributed to the highest value obtained (37). Measurements were always performed by the same skilled operator and the coefficient of variation was 0.8%, determined by repeated measurements in a subgroup of 30 subjects (3 measurement per person in 3 different days within one week). According to the World Health Organization (WHO) criteria, osteopenia and osteoporosis were defined by a T score below – 1 and –2.5 SD respectively for the DEXA technique (22). Using phalangeal OSG, osteopenia and osteoporosis were defined by AD-SoS T score < –1 and < –3.2 SD, according to a recent epidemiological study on more than 10.000 women (27). Potential asymptomatic vertebral compression fractures were investigated by standard spinal radiographs in all patients with BMD T-score < -2.5 SD. AVN was detected by computed tomography or magnetic resonance imaging.

CFU-F assay and stromal layer cultures

Bone remodelling was investigated by *in vitro* CFU-F study in 30 transplanted patients and 20 bone marrow donors. Mononuclear cells from BM (BMMNC) were isolated by density gradient centrifugation using lymphocyte separation medium. BMMNCs were washed by centrifugation at 1200 g for 10 minutes at 20°C with phosphate buffered saline (PBS) containing 2% fetal bovine serum (FCS) for CFU-F assay and stromal cell cultures. For CFU-F assay, BMMNC cells were resuspended at a concentration of 2 $x10^6$/mL in McCoy's 5A modified medium containing 10% FBS with L-glutamine (Mesencult, StemCell Technologies, Vancouver, CA) supplemented with 1 $x10^{-8}$ mol/L dexamethasone (Sigma, St Louis, MO, USA), which allows the recruitment of bone marrow mesenchymal cells to the osteoblastic lineage, and plated in 25 cm^2 tissue culture flasks. Fibroblast colony growth was evaluated after incubation at 37°C, 5% CO_2 for 14 days in a humidified atmosphere. Characteristic fibroblastoid cell aggregates of > 50 cells were scored in situ as CFU-F under an inverted microscope. When needed, osteoblastic differentiation of the colonies was defined by their ability to express ALP activity, and tissue cultures were stained with crystal violet for scoring CFU-F. For marrow stromal layer, 5×10^6/mL BMMNCs were resuspended in a culture medium that consisted of long-term stem cell medium (Myelocult, StemCell Technologies, Vancouver, CA) supplemented with 1 $x10^{-6}$ mol/L hydrocortisone sodium hemisuccinate (Sigma, St. Louis, MO, USA), plated into 25 cm^2 tissue culture flasks and incubated in a humidified atmosphere (37°C, 5% CO_2). On a weekly basis the stromal layer cultures were fed by complete replacement of the medium and analyzed for stromal confluence after 4 –5 weeks. All

cultures were performed in duplicate. Lymphocyte separation medium, HBSS and FCS were purchased from Life Technologies, Gaithersburg, MD, USA.

Statistical analysis

Data are expressed as mean ± SD and ± SEM as appropriate throughout the text and in tables. Analysis of risk factors was performed using Pearsons's correlation coefficient for data expressed by parametric values, and using paired Student's *t*-test for non-parametric variables. After grouping the patients with normal or pathological BMD parameters, χ^2 test was used to assess association with specific clinical features. The linear regression was used to detect correlation between densitometer values and risk factors for bone loss. Statistical significance was considered for $p < 0.05$.

Results

Biochemical parameters at the time of bone density evaluation

Serum Ca, P, creatinine, albumin, urinary Ca excretion and iPTH were within the normal range in all patients and did not differ significantly from control values (**Table 2**). ALP was higher in patients than in controls likely because several patients were affected by liver cGVHD. Mean serum testosterone and LH were normal in all males, whereas FSH resulted elevated in 9 men, suggesting spermatogenesis impairment (**Table 2**). Ovarian insufficiency occurred in all but two (90%) women. Sixteen out of 18 amenorrhoic women were receiving hormone replacement therapy (HRT) at the time of testing and gonadotropin levels were within the normal range in all except three, who were undertreated likely because of reduced intestinal absorption due to mild intestinal cGVHD. Two women were not treated by HRT because of severe liver cGVHD. Osteocalcin levels resulted significantly lower

in patients than in controls (p=0.004), while hydroxyproline excretion did not differ significantly between these two groups.

Table 2. Biochemical evaluation of transplanted patients at time of BMD testing vs controls

Variable	*Normal ranges*	*Patients (n= 41)*	*Controls (n=188)*
Serum:			
Calcium (mmol/L)	2.2-2.6	2.36±0.13	2.34±0.11
Phosphorus (mmol/L)	0.7-1.35	1.08±0.17	1.1±0.18
Alkaline phosphatase (U/L)	98-275	317±143 **	88±20
Creatinine (□mol/L)	<133	87±9	80±10
Albumin (g/dl)	3,6-5,2	4.16±0.36	4.3± 0.3
Osteocalcin (ng/ml)	2-22	13.98±6.3*	15.7± 2.5
iPTH (ng/L)	10-75	39.5± 11.2	36.8± 10.7
FSH (U/L) men/women°	2-15/0.3-9.9	15.5±9.4*/7.5±5	7.5±2.8/6.9±3.4
LH (U/L) men/women°	2-10/0.5-7.7	5.7±2.4/5.2±4	9.5±2.3/10.6±2.9
Testosterone (nmol/L)	10.4-34	19.1±7.3	19.8±7.8
Estradiol (pmol/L)°	147-918	91.8±11*	176±30
Urinary:			
hydroxyproline excretion (μmol/m^2)	60-190	130±41.5	125±16
hydroxyproline/creatinine		9.1	9.5

Values are expressed as the means ± SD. °: during HRT treatment; *: p<0.05; ** p<0.01 vs controls.

Bone mineral density in transplanted patients

The median interval between SCT and bone status analysis was 36.5 months (range, 12-120). At L1-L4 vertebrae, lumbar BMD and Z-scores were significantly lower in patients than in controls (p<0.001). According to WHO criteria, seven patients (17%) had osteopenia (3M, 4F) and 5 (12%) osteoporosis (2F, 3M) at this site. Also femoral BMD values and Z-scores differed significantly between patients and controls (p<0.001), 15 patients (37%) (7M, 8F) having osteopenia and 6 (15%) (3M, 3F) osteoporosis at this site. Phalangeal AD-SoS, Z-scores and UBPI were significantly lower in patients than in controls (p<0.001, all); T-score values were within the range of osteopenia in 25 patients (60%) (10M, 15F) and of osteoporosis in 3 (7%) (2M,1F). When female and male patients were compared separately to their gender-matched controls, a significant decrease in bone density was detected at all skeletal sites evaluated (**Table 3**). OSG detected a significant difference in both Ad-SoS and UBPI. No significant difference in densitometric values was found between male and female patients (**Table 3**). Women with amenorrhea lasting more than 3 months had significantly lower AD-

SoS values (1993±80 *vs* 2073±53 m/s, p=0.04) and Z-scores (-1.81±1.13 *vs* - 0.68±0.77 SD; p=0.04) than women with amenorrhea for less than 3 months, while the difference in UBPI levels (0.59±0.21 *vs* 0.76±0.13; p=0.09) did not reach significance. No difference was found regarding amenorrhea by the DEXA technique.

Patients who had been affected by aGVHD had significantly lower lumbar BMD and Z-scores (-1.45±1.49 *vs* –0.25±0.57 SD) than those without this complication (p=0.01). No significant difference was found in BMD values regarding cGVHD occurrence when any *vs* no cGVHD were considered. AD-SoS resulted significantly lower in patients with extensive cGVHD compared to those not affected by any cGVHD (2030±70 *vs* 2041±39 m/s, p<0.001). Prolonged administration of CsA (>199 days) was associated with lower BMD at lumbar spine (p=0.01) and longer steroid use (>210 days) with lower AD-SoS (p=0.03), while no significant relationship was shown between densitometric values and cumulative dose of immunosuppressive treatments.

When bone mass was evaluated in relation to the time elapsed since transplantation, different findings were obtained depending on the analysis method (**Figure 1**). Patients who were evaluated >36 months after SCT had significantly higher (p< 0.001) lumbar BMD than patients evaluated <36 months. BMD values of the group with longer follow-up period overlapped with those of normal controls (p=0.16). Moreover, lumbar BMD showed a linear correlation with the time elapsed since SCT (p=0.008). On the other hand, no difference was seen in patients evaluated before or after the 36th month since SCT at the FN and phalanxes. No correlation was found between OSG values and months from transplant, and AD-SoS remained low even after more than 6 years. Twelve patients belonging to the group evaluated <36 months after SCT were re-evaluated after an additional period of 12 months: bone loss was 7% and 8% of the baseline value at the LS and FN respectively, while it exceeded 10% at the phalanxes. No significant difference was found in osteocalcin levels after < 3 or >3 years from SCT.

Table 3. Bone mass evaluation in transplanted patients *vs* controls

	Males		*Females*	
	Patients	*Controls*	*Patients*	*Controls*
N	21	98	20	90
Age at evaluation	38.7 ± 10	38 ± 9.8	30.45 ± 8.4	31 ± 9
BMI	26.2 ± 3.26	25.9 ± 3.5	23.4 ± 3.5	23.8 ± 3.5
Lumbar BMD (g/cm^2)	0.924 ± 0.2**	1.09 ± 0.08	0.98 ± 0.12*	1.045 ± 0.1
Z-score	-1.06 ± 1.35**	0.018 ± 0.95	-0.705 ± 1.16**	-0.02 ± 0.089
Femoral neck BMD (g/cm^2)	0.832 ± 0.12**	0.96 ± 0.1	0.763 ± 0.14**	0.884 ± 0.09
Z-score	-0.713 ± 0.912**	0.336 ± 0.86	-1.36 ± 0.726**	-0.02 ± 0.093
Finger AdSoS (m/s)	2020 ± 61**	2118 ± 53	2018 ± 84.5**	2116 ± 50
Z-score	-1.192 ± 0.8**	-0.05 ± 0.45	-1.446 ± 1.19**	-0.048 ± 0.42
UBPI	0.77 ± 0.19**	0.95 ± 0.05	0.636 ± 0.21**	0.91 ± 0.07

Values are expressed as means ± SD. Abbreviations – BMI: body mass index; BMD: bone mineral density; Ad-SoS: amplitude dependent speed of sound; UBPI: Ultrasound bone Profile Index. Symbols - *: $p<0.01$; ** $p<0.001$ *vs* respective controls.

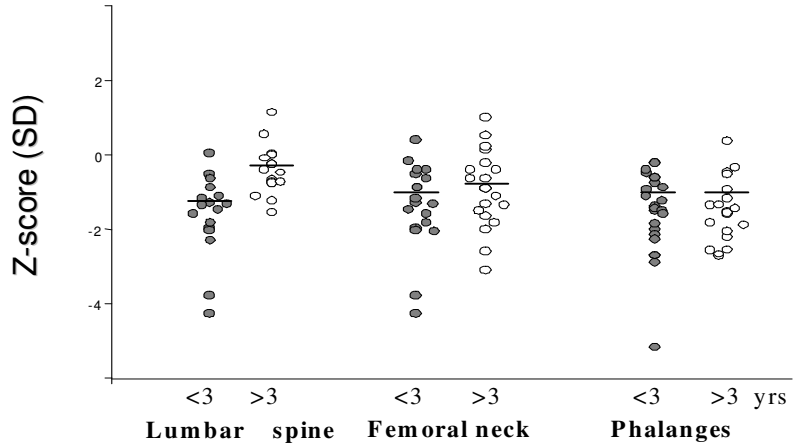

Figure 1. Densitometric values evaluated in patients <3 and >3 years from allogeneic SCT, expressed as Z-score. Each dot represents a subject studied.

Incidence of avascular necrosis and fractures

Eight patients (4F,4M) experienced bone AVN 11 to 120 months after allo-SCT. Sites included both femoral head in all patients with concomitant humeral involvement in three. Clinical features and densitometric values of the subjects with aseptic necrosis are summarized in **Table 4**. Groups with or without AVN were similar in terms of age, follow-up period and BMI. All patients with AVN suffered from extensive cGVHD, having received CsA (p= 0.079) and steroid (p<0.001) treatments for significantly longer periods than patients without this complication. The steroid dose received before AVN occurrence was also significantly higher (p<0.005) than that of patients without this complication. Lumbar and femoral BMD were significantly lower in the group with AVN, while mean AD-SoS value and UBPI did not differ between patients with or without AVN (**Table 4**). However, 6 /8 patients who developed AVN showed AD-SoS T-score value < -1 SD. In this study on a small number of cases with AVN, DEXA measurement was superior to OSG in identifying patients with AVN.

Table 4. Risk factors for avascular necrosis as assessed by the χ^2 test

Variable	*Avascular Necrosis*	*No Necrosis*	χ^2	*P-value*
N	8	33		
Gender: females	4	16		
males	4	17	0.006	0.997
Days on CsA therapy:				
< 199 days	2	18		
≥ 199 days	6	15	2.250	0.325
Cumulative dose of CsA:				
≤60 g	3	23		
>60 g	5	10	2.877	0.237
Days on steroid therapy:				
≤210 days	1	20		
>210 days	7	13	5.964	0.05
Cumulative dose of steroids:				
< 5.8 g	1	20		
≥ 5.8 g	7	13	5.964	0.05
Acute GVHD	5	18		
No acute GVHD	3	15	0.165	0.921
Any chronic GVHD	8	21		
No chronic GVHD	0	12	4.113	0.128
Extensive chronic GVHD	7	12		
No/limited chronic GVHD	1	22	7.063	0.029
Lumbar BMD (g/cm^2)	0.86±0.23	1.01±0.11		0.009*
Femoral neck BMD (g/cm^2)	0.73±0.17	0.823±0.1		0.045*
Phalangeal AD-SoS (m/s)	2019±53	2022±79		0.9*
UBPI	0.72±0.13	0.69±0.22		0.7*

Abbreviations - CsA: cyclosporine A; GVHD: graft versus host disease; BMD: bone mineral density, AD-SoS: amplitude dependent speed of sound; UBPI: ultrasound bone profile index. *: P values were calculated by t-test.

Among osteoporotic patients, multiple non traumatic vertebral fractures were detected in 2 men (age, 52 and 38 yrs) 18 and 30 months after SCT. Both of them were affected by chronic GVHD and had taken > 10 g of prednisone equivalent (for 548 and 510 days) and CsA 51.2 and 32.1 g (for 395 and 240 days).

Marrow CFU-F and stromal layer in transplanted patients

All transplanted patients showed complete engraftment at hemopoietic and molecular level at the time of analysis. Marrow compartment of stromal cells, measured as CFU-F cells, was decreased 2 to 3-fold in transplanted patients compared to normal donors ($22.3\pm3/10^5$ MNC plated *vs* 55±4; p <0.0001) (**Figure 2**). As some of us recently documented for the marrow long-term culture-initiating cell compartment (45), CFU-F cell number in transplanted patients was not influenced by the number of myeloid progenitors (CFU-GM) infused: using as

cut-off the median value ($2.93x10^4$/kg CFU-GM), there was no difference in CFU-F frequency between the groups of patients who had received more or less CFU-GM (p =0.74) (45). To analyze marrow microenvironment at a functional level, we studied the capacity of stromal cells to generate a confluent stromal layer, which is essential to support allogeneic hematopoietic progenitors growth *in vitro*. After 4-5 weeks long-term cultures, marrow stromal cells produced a confluent marrow stroma only in 20% of cases compared to 80% of normal controls (data not shown). Analyzing the effect of time elapsed since transplantation on marrow CFU-F number, we found marrow CFU-F compartment markedly depleted during the first 6 years after transplant (**Figure 2**). Between the 6^{th} and 10^{th} year, the mean marrow CFU-C cell number tended to increase (13.28±3.48 *vs* 33.30±5.36 before and after 67 months, respectively; p=0.004), although most of the patients showed CFU-F numbers permanently below those observed in normal controls. cGVHD was significantly related to decreased number of CFU-F colonies *in vitro* (46.4 ±9.31 vs 13.7±12.85, p=0.01), while no relationship was found in patients with or without avascular necrosis (16.4±18 *vs* 20.2±17).

Variables predictive for bone loss and avascular necrosis

In order to gain insight into the cause of SCT-related bone abnormalities, a univariate analysis was performed to assess the relationship between potential risk factors, densitometric values, AVN occurrence and CFU-F growth *in vitro*. The following risk factors were considered: age, gender, BMI, cumulative dose and length of steroid and CsA received, duration of amenorrhea and a/cGVHD occurrence. Age at SCT correlated inversely with AD-SoS (r= -0.43, p= 0.006), UBPI (r=-0.39, p= 0.01) and lumbar BMD (r= -0.5, p=0.01). A similar correlation was also found with age at bone status evaluation (p= 0.001; p= 0.01 and p=0.02 respectively). Length of amenorrhea correlated with AD-SoS (r= -0.38, p= 0.03). On the other hand, no correlation was shown between densitometric parameters at any site and sex, BMI, immunosuppressive treatments and cGVHD grading. Osteocalcin levels were inversely correlated with UBPI (r=-0.7; p=0.03) and slightly with AD-SoS (r= -0.64; p=0.06). Only extensive cGVHD was strongly related to the onset of AVN, while steroid treatment length and dose resulted borderline (p=0.05, both) (**Table 4**).

CFU-F growth correlated with Ad-SoS, its Z-score (r= 0.48; p= 0.05; r= 0.48, p=0.04, respectively) and with Z-scores of lumbar and FN BMD (r=0.39, p=0.03 and r= 0.55, p<0.04, respectively). The grade of aGVHD did not affect CFU-F number after SCT (p=0.38; p=0.06 and p=0.43 for grade I, II and III-IV). cGVHD alone was related to a significantly lower number of CFU-F colonies *in vitro* (r=0.55; p=0.002).

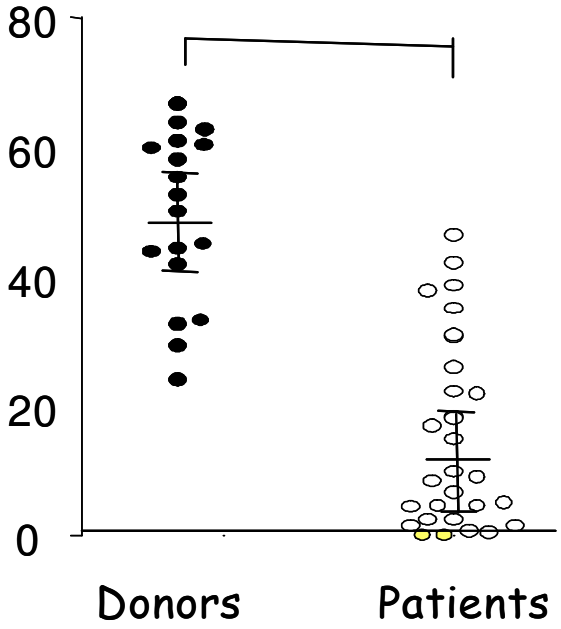

Figure 2. Clonogenic stromal precursors evaluated in patients <3 and >3 years from allogeneic SCT. Bone marrow mononuclear cells (BMMNC) were used for colony forming units-fibroblast (CFU-F) assay. Each dot represents a subject studied.

Discussion

In the last few years, it has became clear that osteoporosis and osteonecrosis represent frequent and serious complications of allo-SCT. As already suggested by other cross-sectional based studies, our results document a frequent decrease in BMD (T-score < -1SD) after allo-SCT at the lumbar spine (29% of patients) and even more at the femoral neck (51%) (10,21,40). A significant decrease in BMD appears early after transplant and seems to continue over the first 3 years with no deterioration afterwards. Only two prospective studies have attempted to establish the exact time when bone loss occurs after SCT. A study by Välimäki *et al*. revealed post transplant bone loss within six months after allogenic SCT at both spine and femoral neck (9). A more recent and larger study by Stern *et al.* (11) found a significant decrease in lumbar and femoral BMD within 3 months, the decrease in femoral BMD continuing (-2.5%) between the 3rd and the 12th month after transplantation (11). Other smaller studies have described bone loss, prevalent at femoral neck, within a few months after SCT (10,21,38-40).

Bone strength is determined by two main features: bone density and bone quality. While bone density accounts for most of the strength, bone quality refers to architecture, turnover, damage accumulation and mineralization (41). The DEXA technique measures bone density and mineralization but does not provide information on architectural damage and bone formation. OSG may detect more physical properties of bone tissue and accounts for more

structural changes than traditional methods (26,27). Ultrasound velocity depends on bone density and elasticity, trabecular orientation and cortical-to-trabecular ratio, all of which are influenced by mineral content and organic matrix (26). By phalangeal OSG (27), 61% of patients could be classified as having osteopenia and 7.3 % as having osteoporosis. Although a positive correlation was found in healthy controls between BMD and phalangeal OSG results, we failed to show any correlation between these two methods after allo-SCT. Such a lack of correlation likely represents further evidence that DEXA and OSG measure different parameters associated with bone loss. In contrast to lumbar densitometry, femoral BMD and ultrasonometric parameters (AD-SoS and UBPI) were persistently decreased in patients suggesting that bone loss may persist for many years after transplantation or may be irreversible.

Multiple atraumatic spine fractures were detected by screening in two patients who were osteoporotic at lumbar spine. AVN occurred in 8 patients after 11 to 120 months since allografting. Men and women were equally affected, all of them having been previously treated with prolonged high dose steroid therapy for extensive cGVHD. Significant statistical association was found by the χ^2 test between AVN occurrence and extensive cGVHD, while dose and length of steroid treatment resulted just at the significance limit (p=0.05).

Discussion on the pathophysiology of post-transplant bone loss always rely on the main contributing factors: age, BMI, immunosuppressive treatment and hypogonadism. As documented both *in vitro* and *in vivo*, steroids may reduce formation and increase resorption of bone, whereas CsA has been shown to increase both bone formation and resorption (44). In addition, the onset of hypogonadism leads to rapid bone loss due to osteoclasts overactivity (22).

A possible recovery in lumbar BMD with time after transplant was suggested by the Pearson's correlation analysis between time elapsed since SCT and lumbar BMD. Among different risk factors for bone loss tested by univariate analysis, age at SCT correlated inversely with spine BMD and phalangeal AD-SoS. The latter was inversely correlated also to amenorrhea length. By the χ^2 test, longer CsA treatment (≥199 days) and aGVHD were associated with lower lumbar BMD values, whereas longer steroid treatment (>210 days) and amenorhea period (> 3 months) were associated with lower OSG values. No association was found between densitometric values and BMI, cumulative doses of steroids and CsA, and cGVHD.

Mesenchymal stem cells residing in bone marrow are progenitors for osteoblasts and other mesenchymal cell lineage. CFU-Fs represent clonogenic mesenchymal progenitors

leading to precursors for stromal microenvironment and osteogenic compartment able to support hemopoiesis and to form bone tissue *in vitro* and *in vivo* (31-33). The origin of post-transplant CFU-F is still controversial; a recent large study suggests a recipient origin (34). Under growth conditions known to promote the maturation of primitive osteogenic precursors in the CFU-F fraction of human bone marrow, the numbers of marrow CFU-F in transplanted patients remains permanently below those observed in normal donors, although some recovery has been observed after 6 years post-SCT. In agreement with a recent report in a small group of patients evaluated for osteoblastic precursors within the first year after SCT (46), we documented that impairment of osteogenic progenitors was severe and persistent after allo-SCT. Furthermore, bone marrow stromal cells of almost all transplanted patients were unable to give rise to confluent stroma in long-term cultures, implying also a long-lasting functional damage. These findings suggest that in transplanted patients the decrease in bone mass could in part reflect a decreased number and function of osteoblasts due to transplant-related loss of osteogenic progenitors. This hypothesis is further supported by the correlation found between low densitometric values and marked decrease in CFU-F growth, and is in line with the finding of persistently low osteocalcin values.

In conclusion, our data confirm and expand the results of previous studies showing a significant decrease in BMD and a reduced functional capacity of osteoblastic precursors after allo-SCT (11,46). Early bone loss may consist of both demineralization and organic matrix deficit, the first detectable by DEXA and the second by OSG. While mineralization seems to improve at trabecular rich sites (LS), no significant change was detected at cortical bone (FN). No improvement was revealed by OSG even after a prolonged follow-up. Phalangeal OSG and DEXA are not interchangeable methods for bone loss estimation. FN and phalangeal OSG may be preferable sites for investigation of transplant-related bone loss in patients who survive more than 3 years from allografting. Whether or not combined measurements from these anatomical sites improve bone loss detection still needs to be determined. Although long-lasting steroid therapy for cGVHD and hypogonadism play major roles in determining bone loss, our study also shows a severe and permanent deficit in number and function of osteoblastic precursors within the stromal stem cell compartment, suggesting that inability to regenerate a normal osteogenic cell compartment may in part account for severe bone damage after allo-SCT.

References

1. Bacigalupo A, Oneto R, Bruno B, Soracco M, Lamparelli T, Gualandi F, Occhini D, Raiola A, Mordini N, Berisso G, Bregante S, Dini G, Lombardi A, Lint MV, Brand R 1999 Early predictors of transplant-related mortality (TRM) after allogeneic bone marrow transplants (BMT): blood urea nitrogen (BUN) and bilirubin. Bone Marrow Transplant 24:653-659
2. Katz IA, Epstein S 1992 Perspectives. Post-transplantation bone disease. J Bone Min Res 7:123-126
3. Apperlay J, Cohen A, Niethammer D, Socié G 2000 Late complications. In: Apperlay JF, Gluckman E, Gratwohl A eds. Blood and marrow transplantation handbook. ESH Paris; 183-199
4. Shane R, Rivas MDC, Silverberg SJ, Kim TS, Staron RB, Bilezikian JP 1993 Osteoporosis after cardiac transplant. Am J Med 94:257-264
5. Lee AH, Mull RL, Keenan GF, Callegari PE, Dalinka MK, Eisen HJ, Mancini DM, Di Sesa VJ, Attie MF 1994 Osteoporosis and bone morbidity in cardiac transplant recipients. Am J Med 86:35-41
6. Julian BA, Laskow BA, Dubovsky J, Dubovsky EV, Curtis JJ, Quarles LD 1991 Rapid loss of vertebral mineral density after renal transplantation. New Engl J Med 325:544-550
7. Keogh JB, Tsalamandris C, Sewell RB, Jones RM, Angus PW, Nyulasi IB, Seeman E 1999 Bone loss at proximal femur and reduced lean mass following liver transplantation: a longitudinal study. Nutrition 15:661-664
8. Kelly PJ, Atkinson K, Warld RL, Sambrook PN, Biggs JC, Eisman JA 1990 Reduced bone mineral density in men and women with allogenic bone marrow transplantation. Transplantation 50:881-882
9. Valimaki MJ, Kinnunen K, Volin L Tahtela R, Loyttyniemi E, Laitinen K, Makela P, Keto P, Ruutu T 1999 A prospective study of bone loss and turnover after allogenic bone marrow transplantation: effect of calcium supplementation with or without calcitonin. Bone Marrow Transplantation 23:355-361
10. Kauppila M, Irjala K, Koskinen P, Pulkki K, Sonninen P, Viikari J, Remes K 1999 Bone mineral density after allogeneic marrow transplantation. Bone Marrow Transplant 24:885-889
11. Stern JM, Sullivan KM, Ott SM, Seidel K, Fink JC, Longton G, Sherrard DJ 2001 Bone density loss after allogeneic hematopoietic stem cell transplantation: a prospective study. Biol Blood Marrow Transplant 7:257-264
12. Schimmer AD, Mah K, Bordeleau L, Cheung A, Ali V, Falconer M, Trus M, Keating A 2001 Decreased bone mineral density is common after autologous blood or marrow transplantation. Bone Marrow Transplantation 28:387-391
13. Weilbaecher KN 2000 Mechanisms of osteoporosis after hematopoietic cell transplantation. Biol Blood Marrow Transplant 6:165-71
14. Mundy GR 1996 Bone resorbing cells. In: Favus MJ ed. Primer on the metabolic bone diseases and disorders of mineral metabolism. Lippincott Raven Philadelphia; 16-24
15. Schimmer AD, Minden MD, Keating A 2000 Osteoporosis after blood and marrow transplantation: clinical aspects. Biol Blood Marrow Transplant 6:175-81
16. Schubert MA, Sullivan KM, Schubert MM, Nims J, Hansen M, Sanders JE, O'Quigley J, Witherspoon RP, Buckner CD, Storb R 1999 Gynecological abnormalities following allogenic bone marrow transplantation. Bone Marrow Transplant 5:425-430
17. National Institute of Health 2001 Consensus Development Conference Statement: Osteoporosis Prevention, Diagnosis, and Therapy. JAMA 285:785-95
18. Withold W, Wolf HH, Kolbach S, Heyll A, Schneider W, Reinauer H 1996 Monitoring of bone metabolism after bone marrow transplantation by measuring two different markers of bone turnover. Eur J Clin Chem Clin Biochem 34:193-197
19. Carlson K, Simonson B, Ljunghall S 1994 Acute effects of high-dose chemotherapy followed by bone marrow transplantation on serum markers of bone metabolism. Calcif Tissue Int 55:408-411
20. Kang MI, Lee WY, Oh KW, Han JH, Song KH, Cha BY, Lee KW, Son HY, Kang SK, Kim CC 2000 The short-term changes of bone mineral metabolism following bone marrow transplantation. Bone 26:275-279
21. Kashyap A, Kandel F, Yamauchi D, Palmer JM, Niland JC, Molina A, Fung H, Bhatia R, Krishnan A, Nademanee A, O'Donnell MR, Parker P, Rodriguez R, Snyder D, Spielberger R, Stein A, Nadler J, Forman SJ

2000 Effects of allogeneic bone marrow transplantation on recipient bone mineral density. A prospective study. Biol Blood Marrow Transplant 6:344-351

22. World Health Organization Study Group 1994 Assessment of fracture risk and its application to screening for postmenopausal osteoporosis. WHO Technical Report Series 843. WHO, Geneve.

23. Beck TJ, Ruff CB, Warden KE, Scott WW Jr, Rao GU 1990 Predicting femoral neck strenght from bone mineral data. A structural approach. Invest Radiol 25:6-18

24. Brinckmann P, Biggemann M, Hilweg D 1989 Prediction of the compressive strength of human lumbar vertebrae. Spine 14:606-610

25. Hayes WC, Piazza SJ. Zysset PK 1991 Biomechanics of fracture risk prediction of the hip and spine by quantitative computed tomography. Radiol Clin North Am 29:1-18

26. Cadossi R, de Terlizzi F, Canè V, Fini M, Wüster C 2000 Assessment of bone architecture with ultrasonometry: experimental and clinical experience. Hormone Research 54 (Suppl 1):9-18

27. Wüster C, Albanese C, De Aloysio D, Duboeuf F, Gambacciani M, Gonnelli S, Gluer CC, Hans D, Joly J, Reginster JY, De Terlizzi F, Cadossi R 2000 Phalangeal Osteosonogrammetry study: age-related changes, diagnostic sensitivity, and discrimination power. J Bone Miner Res 15:1603-1614

28. Sili Scavalli A, Marini M, Spadaro A, Riccieri V, Cremona A, Zoppini A 1996 Comparison of ultrasound transmission velocity with computed metacarpal radiogrammetry and dual-photon absorptiometry. Eur Radiol 6:192-195

29. Kaufmann JJ, Einhorn TA 1993 Perspectives ultrasound assessment of bone. J Bone Min Res 8:517-525

30. Gonnelli S, Cepollaro C, Pondrelli C, Martini S, Monaco R, Gennari C 1997 The usefulness of bone turnover in predicting the response to transdermal estrogen therapy in postmenopausal osteoporosis. J Bone Miner Res 12:624-631

31. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR 1999 Multilineage potential of adult human mesenchymal cells. Science 284:143-147

32. Kuznetsov SA, Krebsbach PH, Satomura K, Kerr J, Riminucci M, Benayahu D, Robey PG 1997 Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo. J Bone Miner Res 12:1335-1347

33. Carlo Stella S, Tabilio A, Regazzi E, Garau D, La Tagliata R, Trasarti S, Andrizzi C, Vignetti M, Meloni G 1997 Effect of chemotherapy for acute myelogenous leukemia on hematopoietic and fibroblast marrow progenitors. Bone Marrow Transplant 20:465-471

34. Galotto M, Berisso G, Delfino L, Podesta M, Ottaggio L, Dallorso S, Dufour C, Ferrara GB, Abbondandolo A, Dini G, Bacigalupo A, Cancedda R, Quarto R 1999 Stromal damage as consequence of high-dose chemo/radiotherapy in bone marrow transplant recipients. Exp Hematol 27:1460-1466

35. del Puente A, Heyse SP, Mandes MG, Mantova D, Carpinelli A, Nutile G, Oriente P 1998 Epidemiology of osteoporosis in women in southern Italy. Aging Clin Exp Res 10:53-58

36. Tauchmanovà L, Rossi R, Nuzzo V, del Puente A, Esposito-del Puente A, Pizzi C, Fonderico F, Lupoli G, Lombardi G 2001 Bone loss determined by quantitative ultrasonometry correlates with the disease activity in patients with different degree of endogenous glucocorticoid excess due to adrenal mass. Europ J Endocrinol 145:237-239

37. Albery A 1982 On the use and computation of likelihood ratios in clinical chemistry. Clin Chem 5:1113-1119

38. Castaneda S, Carmona L, Carvajal I, Carvajal I, Arranz R, Diaz A, Garcia-Vadillo A 1997 Reduction of bone mass in women after bone marrow transplantation. Calcif Tissue Int 60:343-347

39. Kelly PJ, Atkinson K, Ward RL, Sambrook PN, Biggs JC, Eisman JA 1990 Reduced bone mineral density in men and women with allogenic bone marrow transplantation. Transplantation 50:881-883

40. Ebeling PR, Thomas DM, Erbas B, Hopper JL, Szer J, Grigg AP 1999 Mechanisms of bone loss following allogenic and autologous hemopoietic stem cell transplantation. J Bone Miner Res 14:342-350

41. Bauer DC, Glüer CG, Cauley JA, Vogt TM, Ensrud KE, Genant HK, Black DM 1997 Bone ultrasound predicts fractures strongly and independently of densitometry in older women: A prospective study. Arch Intern Med 157 :829-834

42. Montagnani A, Gonnelli S, Cepollaro M, Mangeri M, Monaco R, Bruni D, Gennari C 2000 Quantitative ultrasound at the phalanges in healthy Italian men. Osteoporosis Intern 11:499-504

43. Canalis E 1996 Mechanisms of glucocorticoid action in bone: implications to glucocorticoid-induced osteoporosis. J Clin Endocrinol Metab 81: 3441-3447

44. Cvetkovic M, Mann GN, Romero DF, Liang XG, Ma Y, Jee WS, Epstein S 1994 The deleterious effects of long-term cyclosporine A, cyclosporine G, and FK506 on bone mineral metabolism in vivo. Transplantation 57:1231-1237

45. Selleri C, Maciejewski JP, De Rosa G, Raiola A, Risitano AM, Picardi M, Pezzullo L, Luciano L, Ricci P, Varriale G, Della Cioppa P, Del Vecchio L, Rotoli B 1999 Long-lasting decrease of marrow and circulating long-term culture initiating cells after allogeneic bone marrow transplant. Bone Marrow Transplant 23:1029-1037

46. Lee WY, Cho SW, Oh ES, Oh KW, Lee JM, Yoon KH, Kang MI, Cha BY, Lee KW, Son HY, Kang SK, Kim CC 2002 The Effect of Bone Marrow Transplantation on the Osteoblastic Differentiation of Human Bone Marrow Stromal Cells. J Clin Endocrinol Metab 87: 329-335

Avascular necrosis in long-term survivors after allogeneic or autologous stem cell transplantation: a single centre experience and a review

Introduction

Avascular necrosis (AVN), a condition defined as nontraumatic ischemic necrosis of bone, has been described in 3-41% of patients who had received an organ transplant, femoral head resulting the prevalent localization (1,2). Following organ transplant, AVN is one of the dreadful complications negatively influencing the quality of life.

Attention to the AVN after stem cell transplant (SCT) was first drawn by Atkinson *et al*. in 1987 (3), in a population of 50 patients surviving >2 years. Since then, studies on larger series of patients have been carried on, which pointed out to a number of significant risk factors, such as the primary disease, age, total body irradiation (TBI) and, in particular, corticosteroid administration.

The pathogenesis of AVN is still matter of controversy: it is considered the result of multiple triggering factors such as metabolic disorders, local vascular damage with temporary or permanent loss of blood supply to the bone, increased intraosseous pressure and mechanical stress leading to demineralization, death of trabecular bone and collapse (4-6). The process is mostly progressive, resulting in joint destruction within three to five years if left untreated. Therefore, surgical treatment is frequently requested in these patients because of functional limitation, with further negative psycho-physical consequences.

Although transplanted patients have multiple risk factors, prolonged high doses of steroids appear to be the main cause for developing AVN. On the other hand, AVN is rare in patients with endogenous Cushing's syndrome, despite very high endogenous corti (7).

Allo- and auto-transplanted patients were mostly pooled together in clinical studies on bone complications after SCT, whereas there could be considerable differences between these two settings. Essentially, the differences consist in different grade of immune system involvement and more prolonged use of immunosuppressive treatments in the allogeneic (allo-) setting. Allograft compromises the host immune system more severely than an autograft (auto-), due to the intense immunosuppressive effect of the conditioning regimen (to avoid graft rejection) and to a prolonged treatment by multiple immunosuppressive drugs

needed to avoid the graft-versus-host disease (GVHD). The frequent development of acute or chronic GVHD (aGVHD, cGVHD) after allo-SCT induces additional relevant alterations in the immune system (8). Finally, the patients treated by allo-transplant are generally younger than those auto-transplanted.

Recently, we have found a straight relationship between AVN and cGVHD in a population of long-term survivors after allo-SCT, while corticosteroid treatment duration and cumulative doses were at the border for statistical significance (9). In order to better understand the mechanisms or the precipiting conditions triggering AVN and to design a treatment strategy, we investigated AVN occurrence in a relatively large population of patients who had received auto- or allo-SCT. Clinical and radiographic features of AVN were compared with the type of transplant, clinical course of cGVHD, its treatments, bone mineral density and the repopulating capacity of stromal stem cells after allo-SCT.

Patients and methods

Patients

Of 255 consecutive patients who had received a SCT procedure, 207 were alive and free of malignancy 180 days after transplant and were included in the study. The patients were prospectively evaluated by two skilled hematologists (GDR and CS) up to 12 years after allo- (n=100, 47F and 53M; age range, 21-55 yrs, median, 32) or auto- (n=107; 49F and 58M; age range, 18-59 yrs, median, 39) SCT. Additional data were obtained by reviewing long-term follow-up medical records starting from diagnosis. The primary disease included acute or chronic myeloid leukemia (AML, CML), acute or chronic lymphocytic leukemia (ALL, CLL), Hodgkin's disease (HD) and non-Hodgkin's lymphoma (NHL). Allo-SCT recipients received unmanipulated marrow-derived stem cells from HLA-identical siblings. Auto-SCT patients received unmanipulated marrow cells (n=35) or mobilized peripheral blood stem cells. Mobilization was achieved by chemotherapy and granulocyte colony-stimulating factor at a dose of 16 μg/kg per day by subcutaneous injection. Clinical features of the patients and previous treatment history for the underlying disease are summarized in **Table 1**. Informed consent was obtained by all patients and the study was designed in accordance with the Helsinki II Declaration on human experimentation.

Conditioning regimens, aGVHD prophylaxis and treatment of acute and cGVHD

Both allo- and auto-transplanted patients for acute AML, CML and ALL received the same conditioning regimen, including busulphan (16 mg/kg in 4 days) and cyclophosphamide (120 mg/kg in 2 days) (BUCY2). Patients with HD, NHL and CLL were conditioned with the BEAM protocol, consisting of carmustine (300 mg/m^2 in 1 day), etoposide (200 mg/m^2 in 4 days), cytarabine (400 mg/m^2 in 4 days) and melphalan (140 mg/m^2 in 1 day). After allografting, aGVHD prophylaxis included cyclosporin A (1 mg/kg i.v. from day –1 to +21, then 10 mg/kg for 6 months orally) and short course methotrexate (10 mg/kg for 4 doses). Diagnosis and grading of GVHD were established according to clinical criteria;[8] the clinical diagnosis was confirmed, if indicated, by histopathology of skin, liver or mucous membranes before starting immunosuppressive therapy. Acute GVHD was treated by pulse high-dose methylprednisolone therapy (2-10 mg/kg for 10 days), followed by slow dose tapering in the following 3 months, monitoring patients' clinical conditions. Chronic GVHD was treated by prednisolone at doses of 1-1.5 mg/kg for 1 month, associated with CsA at doses ranging from 4 to 8 mg/kg/day, followed by 25% tapering every month in the following 6 months. Each organ involved in cGVHD was evaluated for response to immunosuppressive therapy using the following criteria: major or minor responses was defined as complete resolution or improvement of cGVHD signs and symptoms, respectively, while on immunosuppressive therapy; no response and progression as no improvement or worsening in any disease signs or symptoms, respectively, despite immunosuppressive therapy; exacerbation as reactivation of cGVHD symptoms after initial improvement while on immunosuppressive therapy. Fifty-six patients had been affected by aGVHD of global grade 1-3 and 58 were affected by cGVHD (25 limited and 33 extensive form). All these patients were treated as stated above for a period ranging 6-24 months (**Table 1**).

Diagnosis of AVN

The suspicion of AVN was posed on clinical ground by the referring physician during the post-transplant follow-up in our institution. Magnetic resonance imaging (MRI) was performed in all patients with suspicion of AVN by anterior-posterior and lateral scans of T1- and T2-weighted images of the involved site. ^{99}Tc labeled methylene bisphoshonate triphasic bone/joint scans were obtained in 6 patients in stage I-II. A staging system developed by ARCO (Association of Research Circulation Osseous) was used (10).

Densitometric evaluation

Bone mineral density (BMD) was performed in a group of 100 patients (50 allo- and 50 auto- transplanted: 46F and 54M; age, 34±10.8 yrs) which was representative of the whole population of long-term survivors and included all patients with AVN. In these patients BMD was evaluated at the moment of AVN diagnosis. Densitometric values were compared with those of 200 healthy subjects matched for age, gender and body mass index (BMI). Lumbar spine (L1-L4) and femoral neck BMD were measured by dual energy X-ray absorptiometry (DEXA), using Hologic QDR 1000 densitometer (Hologic, Inc., Waltham, MA). Individual BMD values were expressed as g/cm^2, T-score and Z-scores.

Colony forming-unit fibroblast (CFU-F) assay

In vitro CFU-F growth was performed as previously described in 30 allotransplanted patients and 20 bone marrow donors (9). Briefly, isolated bone marrow mononuclear cells (BMMNC), after density gradient centrifugation using lymphocyte separation medium (Life Technologies, Gaithersburg, MD, USA), were resuspended at a concentration of 2 $x10^6$/mL in McCoy's 5A modified medium containing 10% FBS with L-glutamine (Mesencult, StemCell Technologies, Vancouver, CA) supplemented with 1 $x10^{-8}$ mol/L dexamethasone (Sigma, St Louis, MO, USA), and plated in 25 cm^2 tissue culture flasks. After incubation at 37°C, 5% CO_2 for 15 days in a humidified atmosphere, characteristic fibroblastoid cell aggregates of >50 cells were scored in situ as CFU-F under inverted microscope. All cultures were performed in duplicate.

Statistical analysis

Data are expressed as mean ± SD or SEM as appropriate throughout the text and in tables. Specific risk factors screened for AVN included gender, age, underlying diagnosis, corticosteroid and cyclosporine exposure, type of transplant, BMD and GVHD occurrence. Analysis of risk factors was performed using Pearsons's correlation coefficient for data expressed by parametric values, and using paired Student's *t*-test for non-parametric variables. After stratifying the patients with and without AVN, χ^2 test was used to assess association with specific clinical features. Linear regression was used to detect correlation between AVN and risk factors. Statistical significance was considered for $p < 0.05$.

Table 1. Characteristics of patients submitted to allogeneic or autologous stem cell transplantation (SCT) and of healthy controls

	Allo-SCT (n= 100)	**Auto-SCT** (n= 107)	**Controls** (n=200)
Age at evaluation (years)	31 ± 10.5	39.6 ± 11.8	34.5±10.4
Time from transplant (months)	38.9 ± 12.2	37.8 ± 12.4	-
F/M	47/53	49/58	97/103
BMI (kg/m^2)	23.4 ± 1.7	25.9 ± 2.1	24.2±1.9
Diagnosis:			
AML	45	50	-
ALL	30		-
CML	10		-
HD	2	35	-
NHL	-	20	-
SAA	3	-	
CLL	-	2	-
***Previous treatment history*:**			
AML	IDA/MITO/ARAC/ETO		
ALL	VCR/DAUNO/CYCLO/MTX/MITO/ARAC/ASP		
CML	HU/ IFN		
HD	VCR/EPIR/BLEO/ETO		
NHL	MTX/ADRI/CYCLO/VCR/PDN		
CLL	CHL/FLUDA		
Corticosteroid dose (g/m^2)	7.2 ± 2.7	7.16 ± 1.4	-
Treatment duration (days)	260.5 ± 32.3*	167 ± 34.8	-
CsA dose (g/m^2)	67 ± 3.42	-	-
Treatment duration (days)	308 ± 39	-	-

Values are expressed as mean ± SD. Corticosteroid cumulative dose is expressed as prednisone equivalents. In the autologous setting, the cumulative doses includes steroids administered before transplant, for treatment of underlying disease. Abbreviations: BMI: body mass index; AML: Acute myeloid leukemia; ALL: acute lymphocytic leukemia; CML: chronic myeloid leukemia; HD: Hodgkin's disease; NHL: non Hodgkin's lymphoma; CLL: chronic lymphocytic leukemia; IDA: idarubicin; MITO: mitoxantrone; ARAC: cytarabine; ETO: etoposide; VNC: vincristine; DAUNO: daunorubicin; CYCLO: cyclophosphamide; MTX: methotrexate; ASP: asparaginase; HU: hydroxyurea; IFN: interferon-γ; EPIR: epirubicine; BLEO: bleomycin, PDN: prednisone; CHL; chlorambucil; FLUDA: fludarabine. *: $p<0.005$ *vs* auto-SCT group

Results

Twelve (7M, 5F) patients developed AVN after SCT: 10 in the allo-SCT group (6M; 4F) and two in the group of auto-transplanted patients (1M, 1F) (83% *vs* 17%; p=0.037). Symptoms of AVN occurred 3 to 120 months following SCT (median, 28) constituted by joint pain (at rest and/or night pain) in 3 patients and joint pain plus functional limitation in 9. A total of 25 joints were affected (mean, 2 joints/person; range, 1 to 4); the joints affected were hip, ankle, shoulder and knee. All patients had lower limb involvement and in 10 patients AVN was confined to lower limbs.

Diagnosis by MRI and scintigraphy

MRI imaging was performed 1-10 months after first symptoms onset. Three patients with unilateral groin pain had bilateral femoral head disease at MRI (controlateral silent involvement). An area of low intensity signal in the femoral head and/or the "double line sign" were observed in nine patients (stage I-II); collapse of femoral head (stage III-IV) was present in three patients. Triphasic scintigraphy, performed in six patients, showed an abnormal uptake (increase uptake surrounding a cold area)(11) in five of them.

AVN in auto-transplanted patients

Two patients developed AVN 3 and 12 months after auto-SCT; the femoral head was affected bilaterally in both. Both patients had hypogonadism: the men was affected by testicular dysgenesis and the woman had post-transplant ovarian failure. Corticosteroid cumulative dose was 7 and 8 g in the patients with AVN and 6.76±0.11 g in autotransplanted patients not affected by this complication. Treatment duration was 240 and 175 days in the two patients with AVN and 167±24.9 days in patients without AVN. Lumbar and femoral Z-scores were -0.03 and –2.39 SD and –1.02 and -0.76 SD, respectively, in patients with AVN, while patients without AVN had lumbar and femoral Z-scores -0.23±0.04 SD and –0.48 ±0.038 SD, respectively.

AVN in allo-transplanted patients

Sites included both femoral heads in 4 patients, one femoral head in three, bilateral femoral head and unilateral/bilateral humeral head in two, bilateral femoral head and right knee in one. AVN developed 12-120 months after SCT. All but one patient affected by AVN were suffering from extensive cGVHD. Exacerbation or recurrence of cGVHD could be

documented in 8 patients shortly (1-4 months) before AVN diagnosis and all these patients had multi-organ involvement. Individual clinical features concerning GVHD of the allo-transplanted patients with AVN are summarized in **Table 2**. Liver manifestation included increased liver enzymes in all patients (ALT, 112-1200 U/L; AST, 67-912 U/L; γGT, 90-350 U/L) and signs of cholestasis (alkaline phosphatase, 487-785 U/L and bilirubin, 5-9 mg/dl) in two. Skin lesions included hypercheratosis, lichen planus and focal epidermal atrophy. Ocular dryness (Sjögren-like syndrome) was the major ophthalmologic manifestation, while oral dryness and lichenoid lesions were the most frequent upper gastrointestinal tract symptoms.

Prior to AVN, patients with this complication had received immunosuppressive treatments for longer periods than allo-transplanted patients who did not develop this complication (CsA, 403±89.3 *vs* 272±35 days; p=0.079; corticosteroid, 448±52 *vs* 207±40 days; p<0.001). The cumulative steroid dose received before AVN occurrence was also significantly higher (15.3±2 *vs* 5.9±1.15 g/m^2; p<0.005) compared with patients without this complication. Two out of four women with osteonecrosis had not received hormone replacement therapy and were hypogonadic since SCT. A T-score < -1 SD at lumbar spine was detected in 4 patients with AVN and at femoral neck in 8, although BMD did not differ significantly between allo-transplanted patients with or without AVN (lumbar, -0.7±0.53 *vs* -0.04±0.3 SD; p=0.065; femoral neck, -1.02±0.5 *vs* –1.07±0.3 SD; p=0.84).

Marrow CFU-F in allo-transplanted patients

Marrow compartment of stromal stem cells, measured by CFU-F assay, was decreased in transplanted patients compared to normal donors (22.3±3/10^5 MNC plated *vs* 55±4; p<0.0001). We found marrow CFU-F compartment markedly depleted particularly in allo-transplanted patients affected by cGVHD (CFU-F: 43.2 ±9.3 *vs* 13.7±12.8 in allo-transplanted patients without and with cGVHD, respectively; p=0.01). In addition, almost all transplanted patients with AVN showed a number of CFU-F below those observed in transplanted patients without this complication (CFU-F: 24.5±3.5 *vs* 12.4 ±4.3 in allo-transplanted patients without and with AVN, respectively; p=0.042).

Table 2. GVHD state in allo-transplanted patients with avascular necrosis (AVN)

Patient	Gender/age at TMO (yr)	AVN onset after SCT (months)	aGVHD grade	CGVHD Form	cGVHD		CGVHD state at AVN diagnosis
					Principal site	Other sites	
1	M/37	54	-	Extensive	Skin	Eyes, oral	stable
2	M/45	53	-	Extensive	Skin	Liver, oral	Exacerbation
3	F/21	10	-	Extensive	Liver	Eyes	Progression
4	F/19	11	1-2	Extensive	Liver	Skin, eyes	Progression
5	M/34	24	4	Extensive	Gut	Skin, liver	Progression
6	F/21	114	-	Extensive	Liver	Skin, eyes	Exacerbation
7	F/35	36	-	Limited	Liver	Eyes	Exacerbation
8	M/19	48	2	Extensive	Liver	Skin, oral	Exacerbation
9	M/36	28	-	Extensive	Skin	Liver, eyes	Progression
10	M/23	18	2	Extensive	Liver	Skin	stable

Time since SCT means the period between SCT and AVN diagnosis

Risk factor assessment

Groups with or without osteonecrosis were similar in terms of age, follow-up period from transplant and BMI. Steroid cumulative dose was similar in the allo- and auto-transplant settings, while the duration of treatment was significantly longer in the allo-transplanted group (p=0.001) (**Table 1**).

Among the risk factors evaluated for AVN by the χ^2 test (**Table 3**), this complication was related to steroid treatment dose and duration (p<0.0001 and p=0.0011, respectively) and to the type of transplant, being significantly more frequent after the allogeneic one (10% vs. 1.9%, χ^2: 6.23; p=0.044). Chronic GVHD, and, in particular its extensive form, was strongly related to AVN occurrence (p=0.018 and p<0.0001, respectively). A recent exacerbation or reactivation of cGVHD could be documented in 8/10 allo-transplanted patients who developed AVN, while other two had mild persistent liver cGVHD (one as extensive and one as limited form). Both lumbar and femoral BMD expressed as Z-score were significantly lower in allo- than in auto- transplanted patients (**Table 4**), suggesting greater bone loss in the allogeneic setting. Although nine out of twelve patients who developed AVN showed a Z-score value at the femoral neck < -1 SD, the BMD difference between the groups with and without AVN did not reach significance at femoral site and was marginal at lumbar spine. No significant influence was found regarding gender and CsA treatment on AVN occurrence.

Figure 1. MRI imaging of a recent bilateral femoral head osteonecrosis in a 39 year old man, who developed pain and functional limitation 2 months prior to imaging. Diffuse focal hypointense lesions in epiphyseal and metaphyseal regions on both sides in coronal T1-weighted image (a), showing high intensity in T2-weighted image (b).

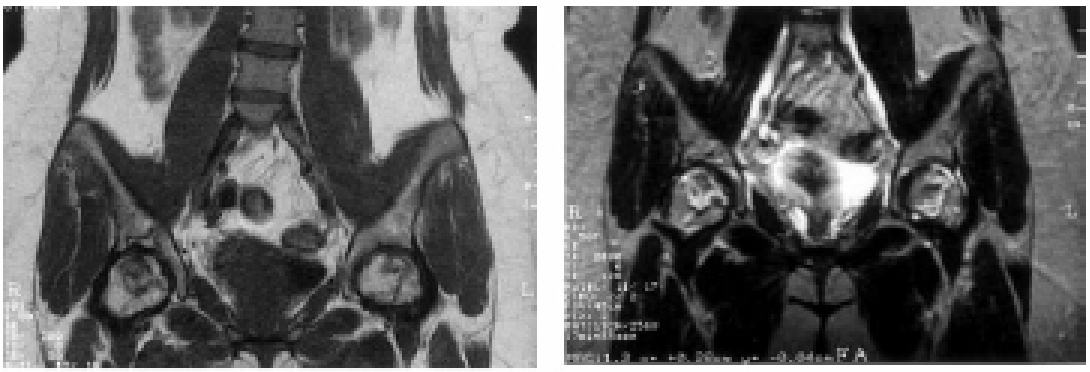

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Table 3. Risk factors for avascular necrosis (AVN) as assessed by χ^2 test

Variable	***+ AVN***	***- AVN***	χ^2	***P*-value**
Number of patients	12	195		
Transplant allogeneic	10	90		
autologous	2	105	6.466	0.039
Gender: Women	5	97		
Men	7	98	0.295	= 0.863
CsA < 199 days*	3	47		
≥ 199 days	7	43	1.9	= 0.385
CsA ≤60 g*	3	47		
> 60 g	7	43	1.907	= 0.385
Steroid duration ≤216 days	2	101		
>216 days	10	94	68.8	< 0.0001
Steroid cumulative dose < 6 g	1	103		
≥ 6 g	11	92	8.95	= 0.0011
Acute GVHD *	6	50		
No acute GVHD*	4	40	0.274	= 0.872
Any chronic GVHD*	10	49		
No chronic GVHD*	0	41	8.05	= 0.018
Extensive chronic GVHD	9	24		
No/limited chronic GVHD	1	66	16.014	<0.0001
Lumbar Z-score <-0.5 DS**	6	44		
>-0.5 DS	6	44	0.0	=1.0
Femoral Z-score <-1.075**	9	41		
>-1.075	3	47	3.41	=0.182
Lumbar BMD (g/cm^2)	*0.97±0.15*	*0.991±0.16*	*t-test*	*=0.667*
Femoral neck BMD (g/cm^2)	*0.78±0.15*	*0.8±0.12*	*t-test*	*=0.598*

*: data relative only to allo-transplanted patients; **: data relative to 50 allo- and 50 auto-transplanted patients; Abbreviations - CsA: cyclosporine A; GVHD: graft versus host disease; BMD: bone mineral density

Treatments

According to the ARCO classification (10), AVN was diagnosed at stage I in five patients, at stage II in four, at stage III in two and at stage IV in one. Conservative treatment with bed rest, analgesics and pulsing electromagnetic fields were adopted in 7 patients in stage I-II, but the disease progressed in three. AVN progression occurred in patients with severe

prolonged GVHD. Six patients with femoral head involvement required surgical treatment. Five total hip replacements were performed in three patients (two bilateral) and core decompression was used in five joints in three subjects (one patient had unilateral replacement and controlateral decompression). Treatments in other localization included rest, analgesics and pulsing electromagnetic fields with significant improvement of clinical symptoms.

Table 4. Bone mineral density measured in a subgroup of patients after allogeneic or autologous stem cell transplant (SCT)

Variable	*Allo-SCT (n=50)*	*Auto-SCT (n=50)*	*P value*
Age (yrs)	29.3 ± 1.6	38.9 ± 1.7	NS
BMI (kg/m^2)	23.5 ± 0.5	25.9 ± 0.6	NS
Femoral neck Z-score (SD)	-1.06 ± 0.14	-0.57 ± 0.17	0.041
Lumbar spine Z-score (SD)	-0.92 ± 0.17	-0.33 ± 0.2	0.017

The results are expressed as mean±SEM; BMI: body mass index.

Discussion

Symptomatic AVN occurred in 12 of 207 long-term survivors after SCT (5.8%) within 3-120 months (median, 28) after grafting, all but two of them having received an allo-transplant. Equal number of men and women were affected. A significant statistical association was found between AVN occurrence, allogeneic type of transplant, presence and grade of cGVHD, steroid treatment length and cumulative dose. These findings are consistent with previous reports on AVN in other series of patients after SCT, their frequencies ranging from 3.7 to 24% (3,12-18). All these studies point out to multiple joint involvement, with prevalent localization at the femoral head. The high M/F ratio (8/1) described in “a total of AVN population” (19,20) appears to be lost in transplanted patients regardless of the type of transplant (21-24). Surgical treatment was required in half of the patients. An additional important information emerged at first by our study is the relationship between AVN and decreased osteoblastic precursor regenerating capacity within the stromal stem cell compartment.

Indeed, the results of the *in vitro* study showed reduced CFU-F growth in patients with AVN. CFU-F assay mirrors the osteoblastic precursor regenerating capacity. Our finding suggests that inability to regenerate a normal osteogenic cell compartment may in part account for AVN onset after allogeneic SCT. A larger number of patients and a

comparison with CFU-F growth in the auto-transplanted setting is needed to confirm this issue. Our study strongly suggests a close relationship between AVN and cGVHD. Indeed, a recent exacerbation or progression of cGVHD could be documented in 8/10 allo-transplanted patients soon before AVN diagnosis. High incidence of acute and/or cGVHD has been previously reported in various studies, pointing out that steroid treatment of this disorder was the most important factor responsible for AVN onset. The relationship between AVN and potential risk factors emerging from previous studies is summarized in **Table 5.** A single report mentioned cGVHD exacerbation as a possible cause of AVN (25), while Enright *et al.* hypothesized an alloreactive immune response as a possible cause for osteonecrosis (12). In a cohort of 902 patients after allo- or auto-SCT followed up between 1974 and 1988, the latter investigator reported an AVN incidence of 10.4% in the allo-transplant setting and 0% in the auto-transplant one; 89% of the allo-transplanted patients with AVN developed GVHD, and 95% had been treated with steroids for GVHD prophylaxis. Older age and GVHD requiring steroid therapy were documented risk factors for AVN among allo-SCT recipients in the largest multicentric study by Sociè *et al*. (14), and corticosteroids resulted as independent risk factor by a multivariate analysis. This study confirmed the results obtained by the same authors in a previous cohort of 727 allo-SCT recipients followed-up in a single center (13), where aGVHD resulted associated with increased incidence of AVN. Similarly, in a study by Fink *et al*. (15) AVN occurred more frequently in the allo- than in the auto-transplant setting (93% *vs* 7%). TBI has been shown as a possible etiologic factor for AVN by various studies, too (12, 14,15). However, similar AVN frequency has been observed in our cohort of patients, none of whom had been treated by TBI.

In any case, the exact mechanisms and risk factors are still unknown for most non-traumatic forms of this invalidating complication. The mechanisms mentioned as potentially contributing for AVN onset include dysregulation of lipid metabolism, drug-induced injury to the vessel wall and vasculitis (4-6,13,20). Lipid metabolism is altered by steroids and gonadal insufficiency, and it has been reported to remain abnormal long after SCT for multifactorial and not completely clarified reasons (X syndrome). Vasculitis after SCT can have autoimmune origin and can be related to cGVHD, as virtually any organ or tissue can be affected by this complication, even in absence of increased autoantibody production. Moreover, AVN is common in conditions of dysregulated immune system, such as the post-transplant period (21-23), SLE (26), but is virtually unknown in other non inflammatory conditions in which long-term high-dose corticosteroids have been used, such as

neurological degenerative disease, chronic pulmonary disease, etc. Furthermore, it has been difficult to reproduce steroid-induces AVN in experimental animal models, unless incompatible skin grafts were applied (27), perhaps simulating the allograft reactivity of the post-transplant state.

Patients with cGVHD are generally those who assume higher steroid dose for longer periods of time. Therefore, on the bases of clinical data only, it is impossible to distinguish the separate effects of these two conditions. Taken into account the available data on general conditions facilitating AVN, the observations on AVN onset after GVHD exacerbation or progression, its relationship with allo- SCT and GVHD documented by this and other studies, we may speculate that immune system dysregulation can be involved in AVN development after SCT.

Cyclosporine A was shown to increase AVN incidence after kidney transplant in a single study (28), while no significant relationship was found in patients after SCT. In our hands, significantly longer CsA treatment was found in patients affected by AVN in the allo-SCT setting.

On the other hand, no relationship was found with gender, age, gonadal function and BMD in the whole patient population. Although significantly lower BMD has been found in allo- than in auto-transplanted patients at both skeletal sites evaluated, no direct relationship could be documented between BMD values and AVN. Despite both auto- and two allotransplanted patients with AVN were hypogonadic for a long period, thus having multiple negative effects on bone status, the lack of AVN relationship with gonadal status in the whole population is in line with a previous evidence that AVN does not seem to be a complication of postmenopausal osteoporosis (19).

Fortunately, most of patients (9/12) were diagnosed in AVN stage I-II, shortly after symptoms appearance. This permitted early treatment intervention. However, surgery was necessary in six patients with hip replacement in three. Early diagnosis by MRI (shortly after the onset of symptoms) may help to detect AVN at an early stage, and early intervention and monitoring may prevent progression to the stages needing prothesic surgery.

In conclusion, our results confirm those of other studies, and focus the attention on several aspects previously incompletely investigated. AVN occurred in 5.8% of our patients, even many years after SCT; allo-transplanted patients with clinical flare up of cGVHD seem to be at highest risk. However, the pathogenesis of this condition is likely multifactorial with various precipitating conditions. An involvement of corticosteroid treatments is pointed out by all previous studies, but the strong relationship between AVN, allo-SCT and cGVHD

relapse documented in this study, suggests that immune-mediated mechanisms may also operate in AVN development after SCT.

References

1. Bewick M, Stewart PH, Rudge C, Farrand C, McColl I. Avascular necrosis of bone in patients undergoing renal allotransplantation. *Clin Nephrol* 1976; 5(2):66-72
2. Hawking KM. Avascular necrosis of bone after renal transplant. Letter. *N Engl J Med*. 1976; 294: 397.
3. Atkinson K, Cohen M, Biggs J. Avascular necrosis of the femoral head secondary to corticosteroid therapy for graft-versus-host disease after marrow transplantation: effective therapy with hip arthroplasty. *Bone Marrow Transplant.* 1987; 2:421-426.
4. Mont MA, Hungerford DS. Non-traumatic avascular necrosis of the femoral head. *J Bone Joint Surg Am.* 1995; 77: 459-474.
5. Chang CC, Greenspan A, Gershwin ME. Osteonecrosis: Current perspectives on pathogenesis and treatment. *Semin Arthritis Rheum.* 1993; 23:47-69.
6. Mankin HJ. Nontraumatic necrosis of bone (osteonecrosis) *N Engl J Med.* 1992;326:1473-1479.
7. Vreden SG, Hermus AR, van Liessum PA, Pieters GF, Smals AG, Kloppenborg PW. Aseptic bone necrosis in patients on glucocorticoid replacement therapy. *Neth J Med.* 1991; 39:153-157.
8. Sullivan KM. Graft versus host disease. In: Forman SJ, Blume KG, Thomas ED, eds. Bone Marrow Transplantation. Boston, MA: Blackwell Scientific Pubblication; 1994: 339-362.
9. Tauchmanovà L, Serio B, del Puente A, et al. Long-lasting bone damage detected by dual energy x-ray absorptiometry, phalangeal osteosonogrammetry and *in vitro* growth of marrow stromal cells after allogeneic stem cell transplantation. *J Clin Endocrinol Metab.* November 2002, in press.
10. Stulberg BN. Editorial Comment on Fifth International Symposium on Bone Circulation. *Clin Ortop.* 1997; 334:2-3.
11. Dumont M, Dunais S, Taillefer R. "Doughnut" sign in avascular necrosis of the bone. *Clin Nucl Med.* 1983; 9:44-47.
12. Enright H, Haake R, Weisdor D. Avascular necrosis of bone: A common serious complication of allogeneic bone marrow transplantation. *Am J Med*. 1990; 89:733-738.
13. Sociè G, Selimi G, Sedel L, Frija J, Devergie A, Esperou Bousdeau H, Ribaud P, Gluckman E. Avascular necrosis of bone after allogeneic bone marrow transplantation: Clinical findings, incidence and risk factors. *Br J Haematol*. 1994; 86:624-628.
14. Sociè G, Cahn JY, Carmelo J, Vernant JP, Jouet JP, Ifrah N, Milpied N, et al. Avascular necrosis of bone after allogeneic bone marrow transplantation: analysis of risk factors of 4388 patients by the Société Francaise de Greffe de Moëlle (SFGM). *Br J Haematol.* 1997; 97:865-870.
15. Fink JC, Leisenring WM, Sullivan KM, Sherrard DJ, Weiss NS. Avascular necrosis following bone marrow transplantation: a case-control study. *Bone*. 1998; 22(1):67-71.
16. Mascarin M, Giavitto M, Zanazzo GA, Andolina M, Cova M, Accorsi E, Tamaro P. Avascular necrosis of bone in children undergoing allogeneic bone marrow transplantation. *Cancer.* 1991; 68:655-659.
17. Wiesmann A, Pereira P, Bohm P, Faul C, Kanz L, Einsele H. Avascular necrosis of bone following allogeneic stem cell transplantation: MR screening and therapeutic options. *Bone Marrow Transplant.* 1998; 22:565-569.
18. Ebeling PR, Thomas DM, Erbans B, Hopper JL, Szer J, Grigg A. Mechanisms of bone loss following allogeneic and autologous hemopoietic stem cell transplantation. J Bone Miner Res. 1999; 14: 342-350.
19. Mazières B. Osteonecrosis. In: Rheumatology; Klippel JH, Dieppe PA. Moshby, London, 1994 pg 47.1-47.10.

20. Calandriello B, Grassi G. Idiopathic osteonecrosis of the femoral head. Epidemiological and aetiological factors. *Ital J Orthop Trauma*. 1982;8:9-18.

21. Shane R, Rivas MDC, Silverberg SJ et al. Osteoporosis after cardiac transplant. *Am J Med*. 1993; 94:257-264.

22. Lee AH, Mull RL, Keenan GF et al. Osteoporosis and bone morbidity in cardiac transplant recipients. *Am J Med*. 1994; 86:35-41.

23. Braun WE, Richmond BJ, Protiva DA, Gifford RW & Straffon RA. The incidence and management of osteoporosis, gout, and avascular necrosis in recipients of renal allografts functioning more than 20 years (level 5A) treated with prednisone and azathioprine. *Transpl Proceed*. 1999;31:1366-1369.

24. Grotz W, Breitffennnldt K, Cybulla M. Immunosuppression and skeletal disorders. *Transplant Proceed.* 2001; 33: 992-993.

25. Mori A, Hashino S, Kobayashi S, Tanaka J, Yamamoto Y, Asaka M, Imamura M. Avascular necrosis in the femoral head secondary to bone marrow infarction in patient with graft-versus-host disease after unrelated bone marrow transplantation. *Ann Hematol*. 2001; 80:238-242.

26. Zizic TM, Marcoux C, Hungerford DS, Dansereau JV, Stevens MB. Corticosteroid therapy associated with ischemic necrosis of bone in systemic lupus erythematosus. *Am J Med.* 1985; 79: 596-604.

27. Gosling DC, Sampson WFD, MadLeod M, Hanby AM, Slapek M. Susceptibiliy of the rabbit and the rat to steroid osteonecrosis: an experimental study. *Transplantation*. 1987; 43: 751-753.

28. Sato K, Satomi S, Oguma S, Ohkoschi N, Okazaki H, Taguchi Y, Mori S. Relationship between aseptic necrosis of femoral head bone and immunosuppression therapy, especially CsA administration. *Nippon Geka Gakkai Zasshi.* 1993; 94:832-839.

29. Kozuch P, Talpaz M, Farderl S, O'Brien S, Freireich EJ, Kantarjian H. Avascular necrosis of the femoral head in chronic myeloid leukemia patients treated with interferon-α. A synergic correlation? *Cancer*. 2000; 89:1482-1489.

30. Wagener P, Schulte D, Link H, Kirchner H, Stoll M, Polwoda H. Muscoloskeletal manifestations in patients after bone marrow transplantation. Initial clinical rheumatologic observation. *Z Rheumatol.* 1991; 50:199-203.

31. Torii Y, Hasegawa Y, Kubo T, Kodera Y, Minami S, Morishita Y, Yamada Y, Iwata H. Osteonecrosis of the femoral head after allogeneic bone marrow transplantation. *Clin Orthop.* 2001; 382:124-132.

Table 5. Risk factors reported by previous major studies on avascular necrosis

Study	**N° of patients**	**Follow up period** (years)	**SCT Type**	**AVN prevalence**	**Assessment of corticosteroid role**	**Associations found with AVN**
Atkinson et al., 1987[3]	50	2	allo	5/50 (10%)	n.r.	AGVHD, cGVHD
Enright et al., 1990[12]	902	14	allo auto	28/642 (4.4%) 0/260	Multivariate analysis	Age, disease type, allo-SCT,TBI, cGVHD, alloreactivity
Wagener et al., 1991[30]	43	3	allo auto	8/33 (24%) 0 /10	t-test	CGVHD, allo-SCT
Sociè et al., 1994[13]	727	1.5	allo	27/727 (3.7%)	Multivariate analysis	Older age, male gender, aGVHD
Sociè et al., 1997[14]	4388	19	allo	77/4388 (17.5%)	Multivariate analysis	Age, initial diagnosis, TBI, aGVHD, cGVHD
Fink et al., 1998[15]	1939	17	allo/auto	87/1939 (5%)	Logistic regression	Allo- (93%) vs auto-(7%) SCT, TBI, aGVHD, cGVHD
Wiesmann et al., 1998[17]	272	n.r.	allo	17/272 (6.3%)	n.r.	Older age, GVHD
Ebeling et al., 1999[18]	83	2.5	allo auto	4/52 (7.7 %) 0/31	t-test	Allo-SCT, male gender, lower BMD at femoral neck
Torii Y et al., 2001[31]	100		allo	19/100 (19%)	t-test	Younger age, cumulative steroid dose, pulse i.v. MPD therapy, cGVHD,

AGVHD, cGVHD: acute/chronic graft-versus host disease; n.r.: data not reported; TBI: total body irradiation; BMD: bone mineral density; MPD: methylprednisolone.

Beneficial treatment with risedronate in long-term survivors after allo-SCT for haematological malignancies

Introduction

Osteoporosis is a relatively common and early complication after allogeneic stem cell transplantation (allo-SCT). The pathogenesis of bone loss related to allo-SCT is complex and still incompletely understood (1). Major risk factors include myeloablative conditioning regimens, huge cytokine release at the time of transplant, long-lasting high-dose steroids and cyclosporin-A (CsA) therapy, reduced mobility and frequent gonadal failure (1,2). Additionally, altered kidney, liver and bowel function result in reduced intake and abnormal metabolism of calcium and vitamin D (1,3). Recently, post-transplant number and function of osteoblastic precursors within the stromal stem cell compartment were found severely and persistently deficient, suggesting that the inability to regenerate a normal osteogenic cell compartment may partly explain the severe bone damage after allogeneic SCT (4-7).

Välimäki *et al.* (8) found that patients undergoing allo-SCT have bone loss at both lumbar spine (5.7%) and femoral neck (6.1-8.6%) after the first six months from allo-SCT. In a larger case series, Stern *et al.* (9) found a significant BMD decrease at lumbar spine and femoral neck even three months after SCT in users of hormone replacement therapy (HRT). BMD further decreased by 2.5% at total hip within 12 months (9). Other studies in smaller cohorts have also described bone loss that was prevalent at the femoral neck within a few months after SCT (10-14). A persistent decrease in femoral BMD was confirmed in two cross-sectional studies evaluating BMD up to 13 years after SCT (4,15).

The treatment of osteoporosis in allo-SCT patients has been poorly investigated. Calcium supplement with or without calcitonin for 12 months did not prevent bone loss within the first year after SCT (8), while increase in lumbar BMD by 9% was found in 13 women treated for 1 year with HRT (16).

Bisphosphonates are currently employed in the treatment of postmenopausal osteoporosis with high success rates (17-19). These compounds are also effective in patients

with severe bone loss such as in endogenous or exogenous hypercortisolism (20-22). Since steroids are widely used in hematological malignancies before and after SCT, bisphosphonates might be considered as the most indicated approach to treat osteoporosis in such patients. Risedronate is a bisphosphonate for oral administration approved in Italy for prevention and treatment of osteoporosis, reported to be well tolerated in long-term treatments (17,20,23,24).

The aim of this open, prospective and randomized study was to investigate the effectiveness of a treatment with risedronate on bone metabolism in allo-SCT recipients.

Patients & Methods

Fifty-five consecutive patients who had undergone allo-SCT in our institution were evaluated for BMD at lumbar spine, total hip and femoral neck, serum osteocalcin levels and urinary hydroxyproline excretion at least 6 months after SCT. Normal BMD values were present in 14 patients, while 41 (22F and 19 M, aged 33±9.5 yrs; range, 20-51) had a BMD T-score at least –1 SD at one or more skeletal sites. Patients with osteoporosis affected by gastrointestinal graft vs. host disease (GVHD) (n= 3) initiated i.v. treatment with zoledronic acid; they were thus not included in this study. Thirty four patients (18 F, 16 M, aged 32±10 yr; range, 20-51) with a T-score < -1.5 SD at lumbar spine and/or femoral neck were enrolled in this study. All of them had been successfully allo-transplanted with unmanipulated marrow from a HLA identical sibling. Reasons for transplant included acute myeloid leukemia (n=14), chronic myeloid leukemia (n=9), acute lymphoblastic leukemia (n=7) and Hodgkin's lymphoma (n=4). All patients had been conditioned with the BU-CY2 regimen (busulphan 16 mg/kg and cyclophosphamide 120 mg/kg) and had received CsA (1 mg/kg/day by continuous i.v. infusion from day -1 to day + 20 and then 8 mg/kg/day orally) plus short-course methotrexate as prophylaxis for GVHD. The median cumulative dose of steroids given to this cohort of patients prior to study entry was equivalent to 5.7 g/kg of prednisone (range 0.8-21.2) for 3-30 months. Eleven patients were continuing glucocorticoid therapy (1-2.5 mg /kg/die of prednisone equivalent) because of persistent chronic GVHD for 2-7 months during the study.

Thirty-four healthy subjects matched for gender, age and body mass index (BMI) were also studied as controls; for ethical reasons, they underwent a single biochemical and densitometric evaluation (**Table 1**). None of controls received drugs interfering with bone

metabolism. Informed consent was obtained from all patients in accordance with institutional guidelines and the study design was made in accordance with the Helsinki II Declaration.

At study entry, bone metabolism, bone density and gonadal status was evaluated in all subjects and BMI in kg/m^2 was calculated. None of the patients had received any previous

Table 1. Clinical, biochemical and densitometric characteristics of patients and controls at study entry.

	Patients (n=34)	**Controls** (n=34)	***P***
Gender (M/F)	16/18	16/18	NS
Age (yr)	32.7 ± 10	32.8 ± 9.5	NS
BMI (kg/m^2)	25.6 ± 2.2	25.5 ± 2.4	NS
Time from SCT (months)	17.5 ± 7	-	
Calcium (mmol/l)	2.36 ± 0.13	2.34 ± 0.11	NS
Phosphorus (mmol/l)	1.08 ± 0.17	1.09 ± 0.18	NS
Alkaline phosphatase (U/l)	91 ± 32	88 ± 20	NS
Creatinine (μmol/l)	86 ± 8.6	80 ± 10	NS
Albumin (g/dl)	4.2 ± 0.36	4.3 ± 0.3	NS
Osteocalcin (ng/ml)	13.9 ± 4.3	15.9 ± 2.5	0.04
iPTH (ng/l)	39.7 ± 10.9	36.9 ± 10.7	NS
Urinary hydroxyproline excretion (μmol/l/m^2)	133.6 ± 32	122 ± 16	0.046
Urinary hydroxyproline/creatinine	9.14 ± 0.8	9.3 ± 0.7	NS
BMD at lumbar spine (g/cm^2)	0.88 ± 0.1	1.05 ± 0.08	<0.001
T - score (SD)	-1.715 ±1.0	-0.2 ± 0.93	<0.001
BMD at total hip (g/cm^2)	0.82 ± 0.08	0.92±0.1	<0.001
T - score (SD)	-1.55 ± 0.78	0.05±0.95	<0.001
BMD at femoral neck (g/cm^2)	0.77 ± 0.1	0.92 ± 0.105	<0.001
T - score (SD)	-1.53 ± 1.0	-0.1 ± 0.9	<0.001

Values are expressed as means ± SD; BMI: body mass index; SCT: stem cell transplantation; BMD: bone mineral density; Normal range, Ca: 2.2-2.6; P: 0.7-1.35; ALP: 98-275 U/L; creatinine: <133 μmol/L; albumin: 3,6-5,2 g/dl; Osteocalcin: 2-22 ng/ml; iPTH: 10-75 ng/L; Hydroxyproline excretion: 60-190μmol/m^2.

treatment for osteopenia/osteoporosis. Then, the patients were randomized to receive treatment with risedronate 5 mg orally once daily at 7.30-8.00 in the morning, at least 30 minutes before breakfast with at least 200 cc of water, calcium 1 g and vitamin D 800 IU once daily orally at 18.00-19.00 daily for six months (n=17, group 1) or the same treatment with calcium and vitamin D without risedronate (n=17, group 2). Randomization was performed according to the program at the website www.randomization.com; the randomization sequence was stored by one of the investigators (A.C.), not directly involved in the treatment nor in the follow-up of the patients, and was inaccessible to the other investigators.

Gonadal status assessment

In all subjects at study entry serum FSH, LH and 17-β-estradiol/testosterone were measured in a single sample at 8.00 a.m. In the patients, but not in the controls, the evaluation was repeated after six months.

Bone metabolism assessment

In both patient groups, at study entry and after 3,6 and 12 months, serum calcium, phosphorus, creatinine, alkaline phosphatase, intact PTH and osteocalcin values were determined in a single blood sample, and urinary calcium, phosphorus and hydroxyproline were assayed in the 24-h urinary collection. Blood samples were collected in the morning after a 12-h fast.

Bone density assessment

In both patient groups, bone density was determined by dual-energy x-ray absorptiometry (DEXA) simultaneously at two different skeletal sites, lumbar spine (L1-L4), total hip and femoral neck (FN), using Hologic QDR 1000 densitometer (Hologic, Inc., Waltham, MA). Individual BMD values were considered as g/cm^2 and T-score. BMD measurement has been performed at baseline and after 6 and 12-13 (median, 12.3) months of treatment. The changes are expressed as a percentage of the baseline value. Quality control was maintained by daily scanning of an anthropomorphic spine phantom. The coefficient of variation was 1.7 % for the lumbar spine and 2.1% for the hip. The reference population adopted in this study was the international pooled sample provided by the manufacturer; their data did not differ significantly from those obtained on a local sample in a study performed when the device was set up (25).

Assays

All measurements were performed by commercially available kits: FSH and LH with radioimmunoassay (RIA, Biodata, Rimini, Italy), testosterone and estradiol using solid phase chemoluminescent enzyme immunoassay (DPC, Los Angeles, CA, USA). Intact PTH and serum osteocalcin levels were measured by RIA (Nichols Institute Diagnostics, CA), the detection limit of the latter being 0.35 mg/L. Hydroxyproline excretion was measured with high pressure liquid chromatography. Blood chemistry profile, including levels of Ca, P, ALP, 24-h urinary Ca excretion and creatinine, were analyzed using a standard autoanalyzer.

Statistical analysis

Data are reported as mean±SD. Statistical analysis was performed by the Student's t test for unpaired data for the intrer-group differences. Friedman's test was used to compare the variables within the same group. Linear regression was used to analyze the relationship between increments of bone density and baseline BMD expressed both as absolute values and Z-score. The significance was set at 5%.

Results

After randomization, both groups were similar in terms of age, time from transplant, BMI, gender (Table 2) and previous treatment history. All men had normal testosterone levels, although it was in the lower tertile of the normal range in five of them. On the other hand, all but one woman had had ovarian failure after SCT. Six female patients (three in each group) were not receiving HRT, while all other women were on standard HRT during whole treatment period, the treatment had been started 6-10 months after SCT. Five patients in the group 1 and six in the group 2 continued corticosteroid treatment, all of them stopped the treatment during the study. Another patient of group 2 initiated steroid plus CsA treatment at 7th month, because of GVHD exacerbation. Similar daily doses were used in both groups (**Table 2**). Cyclosporine A was continued in nine patients (four in group 1 and five in group 2). Steroids were used for 2-7 months (median, 3.6 and 4.1 months for group 1 and 2, respectively) during the study, whereas treatment periods for CsA lasted 3-7.5 months (median, 4.6 and 4.9 months for group 1 and 2, respectively).

Bone metabolism

Serum and urinary calcium, serum phosphorus, creatinine and alkaline phosphatase were normal in all patients both at study entry and during the follow-up (**Table 2**). At baseline, osteocalcin levels were lower (p=0.04) and hydroxyproline excretion higher (p=0.046) in patients than in controls. Urinary hydroxyproline excretion was similar in group 1 and 2 at

Table 2. Variations after 12 month treatment. Group 1 received risedronate, calcium and vitamin D; group 2 only calcium and vitamin D.

	Group 1		Group 2	
	basal	12-months#	Basal	12-months#
Evaluable number (M/F)	8/9	7/8	8/9	8/8
Age (yr)	32.5±10.2		33.2±10.4	
Time since SCT (months)	17±7		18±6	
BMI (kg/m^2)	25.3±2.3	25.15±2.3	25.7±2.2	25.55±2.25
Patients continuing steroid treatment during the study	5	-	6	1
Average daily dose of corticosteroids (mg)[§]	45.6±16	-	55.4±12	12.5
Patients continuing cyclosporine A treatment	4		5	1
Cyclosporine A average daily dose (mg) [§]	119.2±40	-	124±36	50
Women on standard HRT	6/9	5/8	6/9	5/8
Calcium (mmol/l)	2.37±0.12	2.39±0.11	2.36±0.13	2.4±0.12
Phosphorus (mmol/l)	1.07±0.16	1.06±0.2	1.08±0.17	1.07±0.15
Alkaline phosphatase (U/l)	93±25	96±28	90±33	93±28
Creatinine (µmol/l)	86±8.5	88±8	87±9	87.5±9.5
Albumin (g/dl)	4.2±0.4	4.2±0.5	4.15±0.35	4.2±0.4
Osteocalcin (ng/ml)	14±4.4	14.6±5.2	13.9±4.3	14.1±4.1
iPTH (ng/l)	40±10	38.5±9	39.5±11.2	38.4±10.1
Urinary hydroxyproline excretion (µmol/l/m^2)	135±31	113.3±25*	132±33.3	126±34
Urinary hydroxyproline/creatinine	9.2	8.8	9.1	9.0
BMD at lumbar spine (g/cm^2)	0.88±0.075	0.93±0.092*	0.89±0.09	0.864±0.1[a]
T – score (SD)	-1.71±0.85	-1.35 ±0.9	-1.7 ±1.0	-1.85 ±0.95
BMD at total hip (g/cm^2)	0.842±0.05	0.853 ±0.06	0.84±0.08	0.806±0.09 [*a]
T – score (SD)	-1.5 ±0.62	-1.46 ±0.6	-1.49 ±0.7	-1.78 ±0.89
BMD at femoral neck (g/cm^2)	0.77±0.1	0.78±0.1	0.78±0.08	0.747±0.087 [*a]
T – score (SD)	-1.59 ± 1.0	-1.486±0.9	-1.5 ±0.65	-1.8 ±0.8

Values are expressed as means ± SD. #: 15 and 16 patients completed 6 month treatment in the group 1 and 2, respectively; [§:] considered only in patients on treatment; Normal range, Ca: 2.2-2.6; P: 0.7-1.35; ALP: 98-275 U/L; creatinine: <133 µmol/L; albumin: 3,6-5,2 g/dl; Osteocalcin: 2-22 ng/ml; iPTH: 10-75 ng/L; Hydroxyproline excretion: 60-190µmol/m$^{2;}$;*: p<0.05 *vs.* baseline; [a]: p<0.05 *vs*. group 1.

baseline, and decreased by 11.3±3.7% after 3 months (p=0.042) and by 16.8±3.8% (p=0.025) at 12 months in patients of group 1. Serum osteocalcin values were similar in group 1 and 2 at baseline and after 3 and 12 months, without any significant change during the study period (**Table 2**).

Bone mineral density

At study entry, BMD was significantly lower in patients than in controls at each skeletal site (p< 0.001) (**Table 1**). After randomization, BMD was similar in group 1 and group 2 at all skeletal sites considered (**Table 2**). Osteoporosis (T-score <-2.5SD) was found at femoral neck in three patients of group 1 and three of group 2 while at lumbar spine it was found in four of group 1 and in two patients of group 2.

After 6 months, lumbar BMD increased by 4.4±1.6% in patients of group 1 and decreased by 4.3±1.5% in those of group 2 (p<0.05, both). At femoral neck, BMD did not change significantly in patients of group 1, while it decreased in those of group 2; percent BMD change between the two groups was significantly different (1.2±1.2 *vs.* -4.3±2.1%; p<0.05) at this skeletal site. Similar findings were observed at total hip, no change in group 1 and BMD decrease (1.22 ±1.1 *vs.* -4.1±1.6%; p<0.05) in group 2.

After 12 months, lumbar BMD increase achieved 5.9±1.7% (p<0.05) of the baseline value in group 1. In the group 2, BMD increased mildly (1.3±1.4%) between the 6th and the 12th month, reaching - 2.9±1.4% of the baseline value. At femoral neck, BMD did not change significantly in patients of each group between the 6th and the 12th month. However, the percent BMD change between the two groups was significantly different (1.3±1.2 *vs.* -4.2±2.0% from the baseline; p<0.05) at this skeletal site. Similar BMD behavior was observed at total hip (1.35 ±1.1 *vs.* -4.1±1.6% of the baseline; p<0.05) (**Table 2**).

Densitometric values at baseline and after 6 and 12 months are shown in **Figure 1**. The major increase in BMD at lumbar spine has occurred within the first 6 months of treatment, as the mean increase resulted 4.4%, then continuing a slower rise. The percent increment of lumbar BMD during anti-resorptive therapy was inversely correlated with baseline BMD values and Z-scores at the same site (r= -0.71; p=0.049 and r=-0.73*;* p=0.043; respectively).

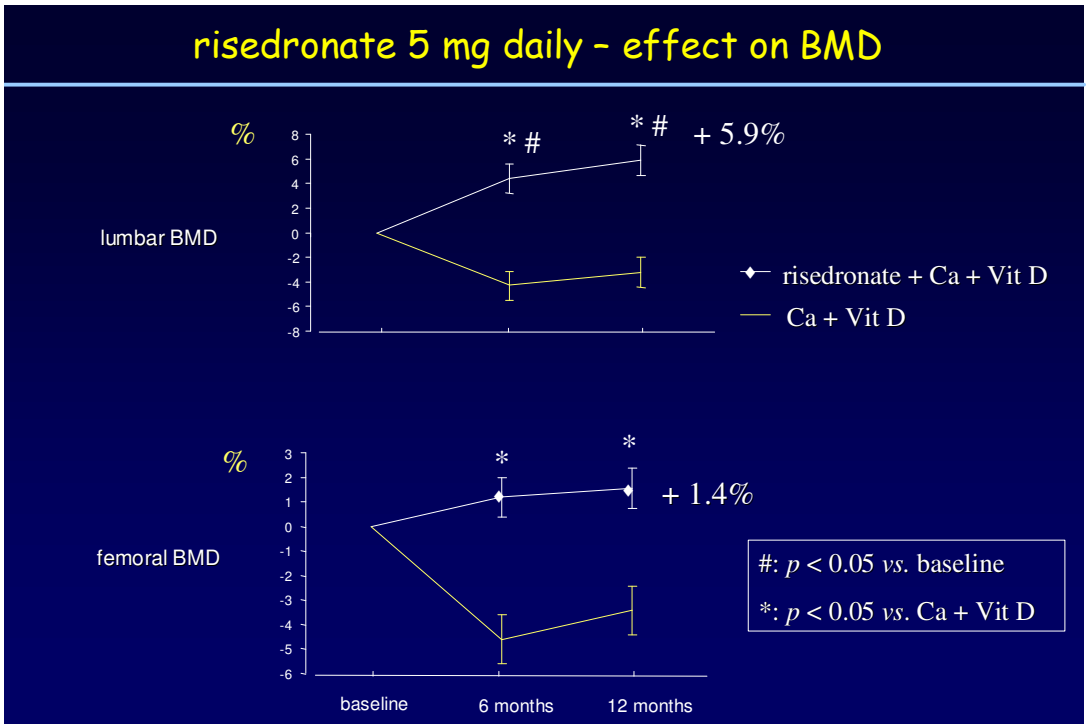


Figure 1. The increase in BMD at lumbar spine after 6 and 12 months of treatment occurred in the treated group, while a decrease was observed in the untreated group at lumbar spine (panel a) and femoral neck (panel b).

Tolerance to treatment

Two patients in group 1 and one patient in group 2 discontinued the treatment because of exacerbation of chronic gastrointestinal GVHD (one in each group) or of gastric intolerance. The treatment was withdrawn after 2-5 months in all three patients. Mild back pain and arthralgia occurred in three and four patients in group 1 and in two and three in group 2. Kidney function parameters did not change in any group over the study period (data not shown).

Discussion

This randomized prospective study demonstrates that oral administration of risedronate at the dose of 5 mg daily for 12 months significantly increased BMD (by 5.9%) at the lumbar spine and prevented bone loss at the femoral neck. Patients treated only by calcium and vitamin

D supplementation lost approximately 4% of bone mass at lumbar spine and femoral neck during the first 6 months, while between the 6th and 12th month of study they realized an increment of 1.3% at lumbar spine. No significant change occurred at femoral neck in the second 6-month period. Nevertheless, changes in BMD at 6 and 12 months were significantly different between the two groups (treated by risedronate or supplementation only) at all skeletal sites.

These data confirm a previous observation of progressive bone loss up to 37 months after allo-SCT (4). Nevertheless, both the initial BMD and subsequent bone loss in the group treated by calcium and vitamin D supplementation were similar at lumbar spine and femoral neck during the first 6 months. These findings confirm those on insufficiency of standard prophylactic measures in prevention of bone loss after allo-SCT (8, 26,27). In this respect, more effective therapeutic strategies may be necessary to prevent bone loss and treat osteoporosis in this peculiar patients' population. It should be noted that the site of prevalent demineralization differ in our patients population compared to the findings of other groups, as femoral neck was the site of prevalent BMD decrease in previous studies (9,15,26). In our cross-sectional study, only the patients evaluated at least 3 years after allo-SCT had a lower BMD at femoral neck: we suggested that this finding is likely due to an improvement of lumbar BMD rather than continuous loss at femoral neck (4). In fact, lumbar BMD started to improve approximately 24 months after SCT in group 2. This is in agreement with a clinical observation that chronic GVHD generally disappear and immunosupressive treatments are withdrawn 24-36 months after transplant. Differences in the populations enrolled in our and other studies can also be taken as partial explanation for the different sites of bone demineralization. In previous studies hypogonadism was replaced early after SCT in all subjects, while six of our female patients were still hypogonadal because of contraindication to HRT or its refusal. To avoid adverse effects, we used to start HRT later (6-10 months after SCT) than previously described in other women, who received HRT as early as 60 days after SCT (9,14,15). Moreover, eleven patients were on low-dose corticosteroid treatment for 2-7 months because of persistent skin, ocular or liver cGVHD that affects about 50% of allo-transplanted patients (28). In this light, our observation is not surprising, as lumbar spine represents an early principal target of bone damage induced by glucocorticoids and gonadal steroids decrease, given its prevalent trabecular structure. Additionally, the spine represents

also a principal target of measurable bone mass improvement during antiresorptive treatments, sexual hormones replacement and general conditions improvement.

Increase in lumbar BMD was greater in our study than that described in menopausal and corticosteroid induced osteoporosis (CIO). Lumbar BMD increased about 3.5-4.5% overt a 12 –month treatment period in postmenopausal women (29-31) and about 2% in patients on chronic steroid treatment (22,32,33). Patients in the CIO studies were on steroid therapy when treated with risedronate. Contrarily, all but one of our patients have withdrawn high-dose (>7.5 mg of prednisone equivalent daily) corticosteroid treatment during the study or shortly before the enrollment. As an increase in lumbar BMD ranging from 14% to 80% (34,35) has been shown within 48 months in patients cured for Cushing's syndrome, we can hypothesize that a part of BMD improvement observed in both groups was due to steroid withdrawal. In our cohort, baseline lumbar BMD was inversely correlated with percent BMD increment after antiresorptive therapy, a finding also observed in patients with CIO (22) and suggesting that the worse is baseline bone density, the greater can be BMD improvement.

The lack of BMD increase at the femoral site during risedronate treatment can be related to the short course of treatment and to the small number of patients included in the active treatment arm. However, no significant BMD increase at femoral neck was also shown in patients with CIO after 1 year of treatment with risedronate (22,32). Additionally, in postmenopausal women the increment of bone density at femoral neck was lower than at lumbar spine after treatment with risedronate (28-30).

Hydroxyproline excretion significantly decreased in group 1 after 3 and 6 months, indicating reduced bone resorption. Indeed, aminobisphosponates act by inhibiting bone resorption (36), although the complete mechanism of action has not yet been clarified. At tissue level, an increase in BMD has been attributed to decreased bone turnover due to reduced frequency and resorption depth of the bone remodeling units (37). At cellular level, bisphosphonates have been shown to have direct and indirect inhibitory effects on osteoclasts (38,39), and to stimulate proliferation and maturation of human osteoblasts. (40). We did not find any significant increase in circulating osteocalcin in patients treated by risedronate, however, this marker of bone formation was lower in patients than in controls at study entry, likely mirroring a decrease in osteoblast number and function previously reported in allogeneic SCT recipients (4-7). Studies investigating osteoblast number and function in allo-

SCT recipients during bisphosphonate treatment may be useful in order to explain the rapid BMD increase. Studies evaluating peri-transplant administration of bisphosphonates in patients undergoing allo-SCT can be usefull.

Aminobisphosponates are widely considered as effective treatment of post-menopausal and glucocorticoid-induced osteoporosis (17,18,20,23,29-33), both conditions present after allo-SCT. Risedronate is likely to improve bone turnover very early, as shown in patients treated for corticosteroid-induced osteoporosis in whom the increase in lumbar BMD was significant even at 3 months (22).

The gonadal status is an independent risk factor for osteoporosis, but the prevalence of women with gonadal insufficiency was similar in each group; due to the small number of cases included in this pilot study, these conditions were not considered separately. Furthermore, male patients were considered to have normal gonadal function, although five of them had testosterone values in the low-normal range. We do not know which were their testosterone values before the onset of the underlying disease and if eventual change in gonadal secretion had contributed to the bone loss.

Risedronate was well tolerated in our patients, and the number of patients who withdrawn because of adverse effects possibly related to the drug (dyspepsia, gastralgia) was minimal (1/17) . These data agree with the results of a previous meta-analysis, showing that daily treatment by 5 mg of risedronate was not associated with increased frequency of adverse gastrointestinal tract effects, even among patients at high risk for these events (24). Back pain and arthralgia, reported also by other authors during the risedronate treatment (22) were mostly self-limited and did not lead to the treatment discontinuation.

The study has several limitations. It was an open label and not blinded study. The follow-up period of 12 months is still relatively short to determine effects on BMD. Moreover, patients have not been evaluated before the transplant to detect the amount of bone loss closely related to the transplant procedure. It should be stated that current main aim of the treatment with biphosphonates is to decrease the fractures rate, the most disabling complication of osteoporosis. However, because of the little size of each treatment arm and relatively short follow-up period, fracture rate was not determined in this study.

In conclusion, the results of this study show that bone loss in long-term survivors to allo-SCT can be stopped and even reversed by risedronate associated with calcium and

vitamin D, while the only administration of calcium and vitamine D is ineffective until GVHD disappears and immunosuppressive treatments are withdrawn. Longer studies are needed to confirm these data and to investigate whether higher doses, different ways of bisphosphonate administration and/or more prolonged treatments time may improve the results.

References

1. Weilbaecher KN. Mechanisms of osteoporosis after hematopoietic cell transplantation. Biol Blood Marrow Transplant 2000;6:165-71.
2. Katz IA, Epstein S. Perspectives. Post-transplantation bone disease. J Bone Min Res 1992;7:123-126.
3. Schimmer AD, Minden MD, Keating A. Osteoporosis after blood and marrow transplantation: clinical aspects. Biol Blood Marrow Transplant 2000;6:175-81.
4. Tauchmanovà L, Serio B, Del Puente A, et al. Long-lasting bone damage detected by dual energy x-ray absorptiometry, phalangeal osteosonogrammetry and *in vitro* growth of marrow stromal cells after allogeneic stem cell transplantation. J Clin Endocrinol Metab 2002;87: 5058-5065.
5. Selleri C, Maciejewski JP, De Rosa G, et al. Long-lasting decrease of marrow and circulating long-term culture initiating cells after allogeneic bone marrow transplant. Bone Marrow Transplant 1991;23:1029-1037.
6. Banfi A, Podesta M, Fazzuoli L, et al. High-dose chemotherapy shows a dose-dependent toxicity to bone marrow osteoprogenitors: a mechanism for post-bone marrow transplantation osteopenia. Cancer 2001; 92:2419-28.
7. Lee WY, Cho SW, Oh ES, et al. The effect of bone marrow transplantation on the osteoblastic differentiation of human bone marrow stromal cells. J Clin Endocrinol Metab 2002;87: 329-335.
8. Valimaki MJ, Kinnunen K, Volin L, et al. A prospective study of bone loss and turnover after allogeneic bone marrow transplantation: effect of calcium supplementation with or without calcitonin. Bone Marrow Transplant 1991;23:355-361.
9. Stern JM, Sullivan KM, Ott SM, et al. Bone density loss after allogeneic hematopoietic stem cell transplantation: a prospective study. Biol Blood Marrow Transplant 2001;7:257-264.
10. Kelly PJ, Atkinson K, Warld RL, Sambrook PN, Biggs JC, Eisman JA Reduced bone mineral density in men and women with allogenic bone marrow transplantation. Transplant 1990; 50:881-882.
11. Kauppila M, Irjala K, Koskinen P, Pulkki K, Sonninen P, Viikari J, Remes K. Bone mineral density after allogeneic marrow transplantation. Bone Marrow Transplant 1999;24:885-889.
12. Kashyap A, Kandel F, Yamauchi D, Palmer JM, Niland JC, Molina A, Fung H, Bhatia R, Krishnan A, Nademanee A, O'Donnell MR, Parker P, Rodriguez R, Snyder D, Spielberger R, Stein A, Nadler J, Forman SJ. 2000 Effects of allogeneic bone marrow transplantation on recipient bone mineral density. A prospective study. Biol Blood Marrow Transplant 6:344-351
13. Castaneda S, Carmona L, Carvajal I, et al. Reduction of bone mass in women after bone marrow transplantation. Calcif Tissue Int 1997;60:343-347.
14. Ebeling PR, Thomas DM, Erbas B, Hopper JL, Szer J, Grigg AP. Mechanisms of bone loss following allogenic and autologous hemopoietic stem cell transplantation. J Bone Miner Res 1999;14:342-350.
15. Buchs N, Helg C, Collao C, et al. Allogeneic bone marrow transplantation is associated with a preferential femoral bone loss. Osteoporos Int 2001; 12:880-886.

16. Castelo-Branco C, Rovira M, Pons F, et al. The effect of hormone replacement therapy on bone mass in patients with ovarian failure due to bone marrow transplantation. Maturitas 1996;23:307-312.

17. Marcus R, Wong M, Heath H III, Stock JL. Antiresorptive treatment of postmenopausal osteoporosis: comparison of study designs and outcomes in large clinical trials with fracture as an endpoint. Endocr Review 2002;23:16-37.

18. Delmas PD. Treatment of postmenopausal osteoporosis. Lancet 2002;8:2018-26.

19. Cranney A, Tugwell P, Adachi J, et al. The Osteoporosis Methodology Group and The Osteoporosis Research Advisory Group. Meta-analysis of Risedronate for the treatmetns of postmenopausal osteoporosis. Endocr Review 2002;23:517-523.

20. Reid DM, Adami S, Devogelaer JP, Chines AA. Risedronate increases bone density and reduces vertebral fracture risk within one year in men on corticosteroid therapy. Calcif Tissue Int 2001;69:242-247.

21. Di Somma C, Colao A, Pivonello R, et al. Effectiveness of chronic treatment with alendronate in the osteoporosis of Cushing's disease. Clin Endocrinol (Oxf) 1998;48:655-662.

22. Cohen S, Levy RM, Keller M, et al. Risedronate therapy prevents corticosteroid-induced bone loss. Arthritis &Rheumatism 1999;42:2309-2318.

23. Watts NB. Risedronate for the prevention and treatment of postmenopausal osteoporosis: results from recent clinical trials. Osteoporosis Int 2001;12 (Suppl 3):S17-22.

24. Taggart H, Bolognese MA, Lindsay R, et al. Upper gastrointestinal tract safety of risedronate: a pooled analysis of 9 clinical trials. Mayo Clin Proc 2002;77:262-70.

25. del Puente A, Heyse SP, Mandes MG, et al. Epidemiology of osteoporosis in women in southern Italy. Aging Clin Exp Res 1998;10:53-58.

26. Schulte C, Beelen DW, Schaefer UW, Mann K. Bone loss in long-term survivors after transplantation of hematopoietic stem cells: a prospective study. Osteoporos Int 2000;11:344-353.

27. Massenkeil G, Fiene C, Rosan O, Michael R, Reisinger W, Arnold R. Loss of bone mass and vitamin D deficiency after hematopoietic stem cell transplantation: standard prophylactic measures fail to prevent osteoporosis. Leukemia 2001;15:1701-5.

28. Sullivan KM, Agura E, Anasetti C, et al. Chronic graft-versus-host-disease and other late complications of bone marrow transplantation. Semin Hematol 1991;28:250-259.

29. Reginster JY. Risedronate increases bone mineral density and reduces the vertebral fracture incidence in postmenopausal women. Clin Exp Rheumatol 2001;19:121-122.

30. Fogelman I, Ribot C, Smith R, et al. Risedronate reverses bone loss in postmenopausal women with low bone mass: results from a multinational, double-blind, placebo-controlled trial. J Clin Endocrinol Metab 2000;85:1895-1900.

31. Reginster JY, Minne HW, Sorensen OH, et al. Randomized trial of the effects of risedronate on vertebral fractures in women with established postmenopausal osteoporosis. Osteoporos Int 2000;11:83-91.

32. Wallach S, Cohen S, Reid DM, Hughes RA, Hoskins DJ, Laan RF, Doherty, Maricic M, Rosen C, Brown J, Barton I, Chines AA. Effects of risedronate treatment on bone density and vertebral fracture in patients on corticosteroid therapy. Calcif Tiss Int 2000;67:277-285.

33. Reid DM, Hughes RA, Laan R, et al. Efficacy and safety of daily risedronate in the treatment of corticosteroid-induced osteoporosis in men and women: a randomized trial. J Bone Miner Res 2000;15:1006-1013.

34. Di Somma C, Pivonello R, Loche S, Faggiano A, Klain M, Salvatore M, Lombardi G, Colao A. Effect of 2 years of cortisol normalization on the impaired bone mass and turnover in adolescent and adult patients with Cushing's disease: a prospective study. Clin Endocrinol 2003;58:302-308.

35. Catargi B, Tabarin A, Basse-Cathalinant B, Ducassou D, Roger P. Development of bone mineral density after cure of Cushing‘s syndrome. Ann Endocrinol (Paris); 1996;57:203-208.

36. Fleisch H. Bisphophonates: mechanisms of action. Endocr Rev 1998;19:80-100.

37. Boyce RW, Paddock CL, Gleason JR, Sletsems WK, Eriksen EF. The effects of risedronate on canine cancellous bone remodeling: three-dimensional kinetic reconstruction of the remodeling site. J Bone Miner Res 1995;10:211-221.

38. Rogers MJ, Chilton KM, Coxon FP, et al. Bisphophonates induce apoptosis in mouse macrophage-like cells in vitro by a nitric oxide-dependent mechanism. J Bone Miner Res 1996;11:1482-1491.

39. Selander K, Lebenkcari P, Väänänen HK. Ä. The effects of bisphophonates on the resorption cycle of isolated osteoclasts. Calcif Tissue Int 1994;55:368-375.

40. Fromigue O, Body JJ. Bisphosphonates influence the proliferation and the maturation of normal human osteoblasts. J Endocrinol Invest 2002; 25:539-546.

Short-term zoledronic acid treatment increases bone mineral density and marrow clonogenic fibroblast progenitors after allogeneic stem cell transplantation

Introduction

Bone loss is recognized as one of the most frequent complications in long-term survivors after allogeneic stem cell transplantation (allo-SCT) (1-5). However, only little data is available so far on the treatment and prevention of this condition (6-8). Early diagnosis of osteoporosis or fast bone loss in this particular population is a major aim to promptly start appropriate supportive measures, such as lifestyle modification, calcium and vitamin D supplementation or antiresorptive therapies.

Oral bisphosphonates are widely used for treating osteoporosis; they have been shown to improve bone mineral density (BMD) and decrease the rate of fractures in various patient populations (9-12). However, the use of these drugs may be limited by poor gastrointestinal tolerance, variable oral bioavailability and long-term compliance. Allo-SCT patients usually run into long-lasting and complex therapeutic regimens in order to prevent and treat several early and late complications related to the transplant procedure. About half of SCT patients suffer from graft-versus-host disease (GVHD), with the gastrointestinal tract being one of the most frequent targets. For these patients, who may have reduced drug absorption, intermittent intravenous administration of bisphosphonates may be the treatment of choice. Zoledronic acid, a third-generation nitrogen-containing bisphosphonates, is the most potent member of the bisphosphonates' family ever investigated in clinical trials (13). Due to its high potency, small doses and long in-between intervals are sufficient enough to inhibit bone resorption, and can easily be added to a complex therapeutic regimen in the post-transplant period.
Intravenous bisphosphonates have proven to be very effective in the treatment of menopausal and glucocorticoid-induced osteoporosis, malignant hypercalcemia and Paget's disease (14-19). Furthermore, the clinical benefit in patients with cancer and multiple myeloma includes bone pain improvement, reduction in skeletal complications and delay in time-to-first skeletal complication (14,19).

Previously, we have shown that bone loss continues for three years after allo-SCT at both the lumbar spine (LS) and femoral neck (FN); it persisted up to 10 years at FN, while

improving mildly but significantly with time at LS (20). We have also documented that post-SCT bone damage is at least in part related to a severe and long-lasting deficit in the regenerating capacity of bone marrow stromal stem cells (20,21).

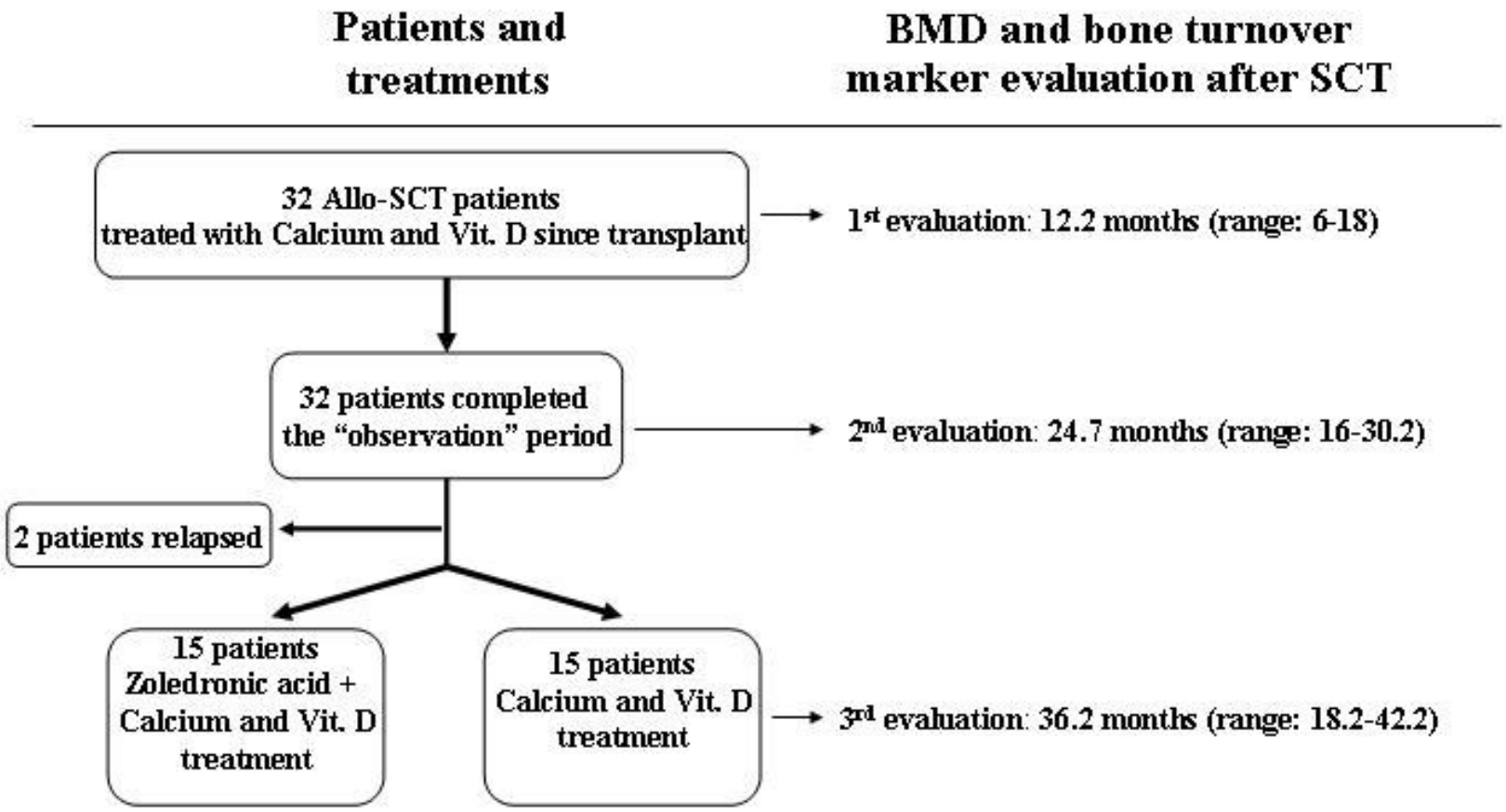
The present study was aimed at investigating the 12-month effect of short-term intravenous zoledronic acid administration on lumbar and femoral BMD, bone turnover markers and clonogenic fibroblast progenitors belonging to the osteogenic stromal lineage, in patients with low BMD or fast bone loss following allo-SCT for hematological malignancies.

Patients and methods

Patients and study design

Thirty-two patients who had undergone allogeneic SCT at our Institution were evaluated for BMD at the LS and FN, body mass index (BMI, kg/m^2), gonadal status, serum osteocalcin and urinary hydroxyproline excretion, after a median period of 12.2 months (range, 6-18) since transplant. Immediately following the transplant, Calcium 500 mg and Vitamin D 400 IU supplementation had been administered orally and once daily to all patients, between 07:00 p.m. and 08:00 p.m. and was continued for the whole observation period. The determination of densitometry and bone turnover markers was repeated after a median time span of 12.5 months since the first evaluation (range, 10.3-13.1). Thus, the median "observation" period was about 1 year. Subsequently, 15 patients (group 1) affected by osteoporosis (n=7) or rapidly progressing osteopenia (i.e. bone loss > 5%/year) (n=8) received 4 mg of zoledronic acid (ZOMETA; Novartis Pharmaceuticals Corporation, Basel, Switzerland) intravenously as a 15 minute infusion every 28 days for three months; seven patients in this group were suffering from extensive cGVHD. Other 15 patients (group 2), who were similar to the zoledronic acid cohort in terms of age, time since transplant, BMI and gender, were re-evaluated for bone turnover and BMD after the same span of time without receiving zoledronic acid. Two patients with Hodgkin's disease who relapsed 13 and 15 months after allo-SCT were excluded from the study (**Figure 1**). To better point out the importance of bone loss within the first year after allografting, two healthy subjects for each case, matched by gender, age and BMI were enrolled as controls, undergoing a single biochemical and densitometric evaluation. None of the controls received drugs known to interfere with bone metabolism. None of the participants had been given previous treatments for osteoporosis. Written informed consent was obtained from all patients and controls in

Figure 1. Algorithm of study design.



accordance with the institutional guidelines and the study was designed in accordance with the Helsinki II Declaration on treatment of human subjects.

Table 1. Clinical features of 32 transplanted patients evaluated for bone mineral density changes

Patient characteristics	Whole group (n=32)
Age at SCT (yrs)	31.4±12
Range	(17 - 45)
Gender (F/M)	15/17
Underlying disease:	
AML in 1st CR	14
CML in chronic phase	12
ALL in 2nd CR	3
HD	2
SAA	1
Amenorrhea duration (months)	24±20
Acute GVHD	14
Grade I-II/III-IV	10/4
Chronic GVHD	17
Limited/extensive form	7/10
Corticosteroid treatment#	
Duration (days)	248±190
Cumulative dose (g)*	5.8±4
Cyclosporin A treatment#	
Duration (days)	338 ±180
Cumulative dose (g)	64.2±12

Values are expressed as mean ± SD, when appropriate. Abbreviations: SCT: stem cell transplant, AML: acute myeloid leukemia, CML: chronic myeloid leukemia, ALL: acute lymphoblastic leukemia, HD: Hodgkin's disease, SAA: severe aplastic anemia, CR: complete remission, BU: busulphan, CY: cyclophosphamide, BMI: body mass index; GVHD: graft versus host disease; Symbols: #: immunosuppressive treatments before study enrollment;*: cumulative dose of corticosteroids is expressed as prednisone equivalent.

Transplantation procedures

Leukemia was the most common indication for allo-SCT. All patients had been successfully allo-transplanted with unmanipulated marrow from a HLA identical sibling. All patients underwent conditioning with the BU-CY2 regimen (busulphan 16 mg/kg, cyclophosphamide 120 mg/kg) and received cyclosporine A (CsA) (1 mg/kg/day by continuous i.v. infusion from day -1 to day + 20 and then 8 mg/kg/day orally) plus short-course methotrexate (10 mg/m^2 on days +1,+3 and +6) as prophylaxis for GVHD. No patient had received radiation therapy. All patients with acute and chronic GVHD (a- and

cGVHD) were treated by methylprednisone (2 mg/kg/day and 1 mg/kg/day for 15 days in patients with aGVHD and cGVHD, respectively) and CsA (5 mg/kg/day) before the first evaluation of bone status. During the study, 53% of patients developed or had persistent cGVHD requiring immunosuppressive therapy as above described. As a significant correlation was found between corticosteroid treatment and bone damage at the initial evaluation, steroids were avoided whenever possible thereafter. During the study period, cGVHD was treated by CsA + mycophenolate mofetil at a dose of 30 mg/kg/day; low-dose corticosteroids were continued in addition to these immunosuppressants in 8 patients only. The median cumulative dose of steroids given to this cohort of patients prior to entry was equivalent to 6.5 g of prednisone (range 0.8-22) for 3-36 months, while it was 1.5+0.8 g for 2.5-5 months during the study. The median daily dose of prednisone equivalents administered during the study was 17.5 mg (range, 5-50 mg).

Gonadal status assessment

At study entry all subjects underwent serum FSH, LH, 17-β-estradiol/testosterone determination in a single sample at 08:00 a.m. In patients only, the same parameters were again evaluated every six months. All women were in the reproductive age. All measurements were performed by commercially available kits: FSH and LH with radioimmunoassay (RIA, Biodata, Rimini, Italy), testosterone and estradiol using solid phase chemoluminescent enzyme immunoassay (DPC, Los Angeles, CA, USA). All controls were eugonadal, females having regular menstrual cycles.

Bone metabolism assessment

Serum calcium, phosphorus, creatinine, alkaline phosphatase (ALP), intact parathyroid hormone (iPTH) and osteocalcin were determined in blood samples drawn at initial evaluation, at the end of the longitudinal study, before zoledronic acid administration and after 12 months. Blood samples were collected in the morning after a 12-h fast. Urinary calcium, phosphorus and hydroxyproline were assayed on the 24-h urinary collection. Standard dietary restrictions were observed by all individual tested, starting five days prior to urinary collection. Intact PTH and serum osteocalcin levels were measured by RIA (Nichols Institute Diagnostics, CA), the detection limit of the latter being 0.35 mg/L. Bone alkaline phosphatase was assayed by a direct immunoradiometric assay (Ostease, Hybritech, San Diego, CA). Hydroxyproline

excretion was measured with high pressure liquid chromatography. Blood chemistry profile, including Ca, P, ALP, urinary Ca excretion and creatinine levels, were analyzed using a standard autoanalyzer.

Bone density assessment

Bone density was determined by dual-energy x-ray absorptiometry (DEXA) at the lumbar spine (L1-L4) and femoral neck (FN), using Hologic QDR 1000 densitometer (Hologic, Inc., Waltham, MA). Individual BMD values were expressed as g/cm^2 and Z-scores. Quality control was performed by daily scanning of an anthropomorphic spine phantom. The coefficient of variation was 1.7% for the LS and 2.1% for the FN. The reference population adopted in this study was the international pooled sample provided by the manufacturer; their data, however, did not differ significantly from those obtained on a local sample in a study performed when the device was set up (23).

Mesenchymal stem cell enrichment

Bone marrow was aspirated from the posterior iliac crest of 20 transplanted patients and 15 bone marrow donors into syringes containing heparin (O'Neil & Feldman, St Louis, MO). Briefly, mesenchymal stem cells were enriched by incubating whole bone marrow with a cocktail of antibodies against glycophoryn A, CD3, CD14, CD19, CD38 and CD66b for 20 minutes at 4°C, according to the manufacturer's instructions (Rosette-Sep, StemCell Technologies Inc., Vancouver, Canada). Unagglutinated cells, following density gradient centrifugation using lymphocyte separation medium (Life Technologies, Gaithesburg, MD), were washed with Hanks' balanced salt solution (HBSS) supplemented with 1% bovine serum albumin (BSA), and resuspended in HBSS 1% BSA. HBSS and BSA were purchased from Life Technologies, Gaithersburg, MD.

Colony forming unit-fibroblast (CFU-F) assay

In vitro CFU-F growth assay was performed using enriched mesenchymal stem cells as previously described (21,22). Briefly, enriched mesenchymal stem cells were resuspended at a concentration of $1x\ 10^6$/ml in a specific medium (McCoy's 5A modified

medium containing 10% fetal bovine serum with L-glutamine) formulated for optimal mesenchymal stem cell expansion (Mesencult, StemCell Technologies) supplemented with 10^{-8} M dexamethasone (Sigma-Aldrich), allowing the recruitment of bone marrow mesenchymal cells to the osteoblastic lineage, and seeded at $4x10^5$ cells/cm^2 in 35-mm tissue culture plates. Fibroblast colony growth was evaluated after incubation at 37°C, 5% CO_2 for 14 days in a humidified atmosphere. Aggregates of >50 characteristic fibroblastoid cells were scored in situ as CFU-F under inverted microscope. Osteogenic differentiation of fibroblastoid colonies was further defined by their ability to express phosphatase alkaline (ALP) activity after replating for 14 days at 5% CO_2 in mesencult medium supplemented with 10^{-8} M dexamethasone, 0.2 mM ascorbic acid and 10 mM β-glycerol phosphate (Sigma-Aldrich) and re-adhesion to plastic ware. All cultures were performed in triplicate.

Statistical analysis

Data are reported as mean±SD unless otherwise specified. Statistical analysis was performed by the Student's *t*-test for paired and unpaired data, as appropriate, for the comparison between patients and controls, and groups of treated and untreated patients. In the period prior to treatment, the association between BMD changes, immunosuppressive treatment duration and cumulative doses, and amenorrhea period were assessed by linear regression. Linear regression analysis was also used to analyze the relationship between increments of bone density and baseline BMD. Comparison of the variables within the same group was performed by the Friedman test. The significance was set at 5%.

Results

Clinical features and gonadal status

In table 1 we have summarized the clinical features, underlying malignancies and details on previous treatments of the population of 32 patients at study entry. Ninety percent of women experienced ovarian failure (gonadotropin levels in the menopausal range and estradiol below the normal range of fertile women) with amenorrhea lasting at least 6 months as a consequence of chemotherapy. Eight women (53.3%) were receiving

hormone replacement therapy (HRT) at the time of the first testing: this treatment had been started 6-12 months after SCT and was continued over the whole study period. The other women who were contraindicated or refused, were not given any HRT until the end of study. HRT consisted of oral administration of estradiol 2 mg/daily and dihydrogesterone 10 mg/day for 14 days a month (Femoston 2/10; Solvey Pharma SpA, Grugliasco, TO, Italia).

In men, testosterone below the normal range with or without LH increase, was considered as a sign of hypogonadism. Eight men were hypogonadal at the initial evaluation; all of them being on immunosuppressive treatment for 4-8 months during the observation. Periods of immunosuppressive treatment mostly coincided with the finding of transient mildly low testosterone levels.

Longitudinal evaluation of BMD after SCT

There were no significant changes in the biochemical parameters of bone turnover during the "observation" period (Table 2). Baseline BMD values in the 32 patients were significantly lower than those of controls at both skeletal sites ($P<0.001$)(Table 2). According to the WHO criteria (22), osteoporosis was diagnosed in 6 patients (19%) and osteopenia in 10 (31%).

In the whole cohort of 32 patients, the average BMD loss per year was 3.42% at LS and 3.8% at FN (**Table 2**). Although bone loss in male and female patients was not significantly different, males tended to lose more bone at FN (-6.4±7.4% vs.-2±8.33%), while bone loss in women was higher at LS (-4.16±6.3 vs. –2.41±8.56).

In the whole group of 32 patients at the first evaluation, adjusted femoral BMD values correlated with age ($r= -0.6$; $P= 0.045$) and BMI ($r= 0.46$; $P=0.013$). At the second evaluation, femoral BMD continued to be significantly associated with BMI ($r= 0.465$; $P=0.017$), but not with age. In women, lumbar BMD correlated inversely with the duration of amenorrhea both at the first and second evaluation. A borderline correlation was found between femoral BMD decrease and BMI ($r=-0.41$; $P=0.05$), suggesting a mild protective effect of weight on FN BMD. A mild but not significant correlation was present between the amount of lumbar BMD decrease and cumulative dose of corticosteroids ($r=-0.44$; $P=0.067$). Serum osteocalcin was slightly correlated with baseline lumbar BMD ($r=-0.62$; $P=0.055$).

At study entry, lumbar BMD was lower in patients with cGVHD compared to patients without this complication ($P<0.05$), while the difference was not revealed at the

second evaluation. The amount of bone loss during the observation period was greater in patients with cGVHD, but not significantly different from those without cGVHD (data not shown). A correlation at the very limit of significance was detected between CsA treatment duration and the amount of FN BMD decrease (r=0.24; p=0.05).

Effects of zoledronic acid on bone turnover and BMD after allo-SCT

During the treatment period, there was a significant decrease in hydroxyproline excretion (P<0.0001) and an increase in bone alkaline phosphatase (p<0.05); in addition, there was a trend towards a negative correlation between serum osteocalcin and baseline lumbar spine BMD, but this correlation did not quite reach statistical significance (r=-0.62; P=0.055) (Table 3). Twelve months after the beginning of the treatment, an increase in BMD was observed at both LS and FN (9.8% and 6.47%, P<0.001 and P <0.005, respectively vs. baseline) (**Figure 2**).

Figure 2. Densitometric values at lumbar spine and femoral neck before and 12 months after treatment with zoledronic acid, expressed as Z-scores. The increase was significant at both lumbar spine (9.8±7%; *p*<0.001) and femoral neck (6.47±7%; *p*<0.005). Horizontal bars represent mean values, vertical bars are the standard error of mean.

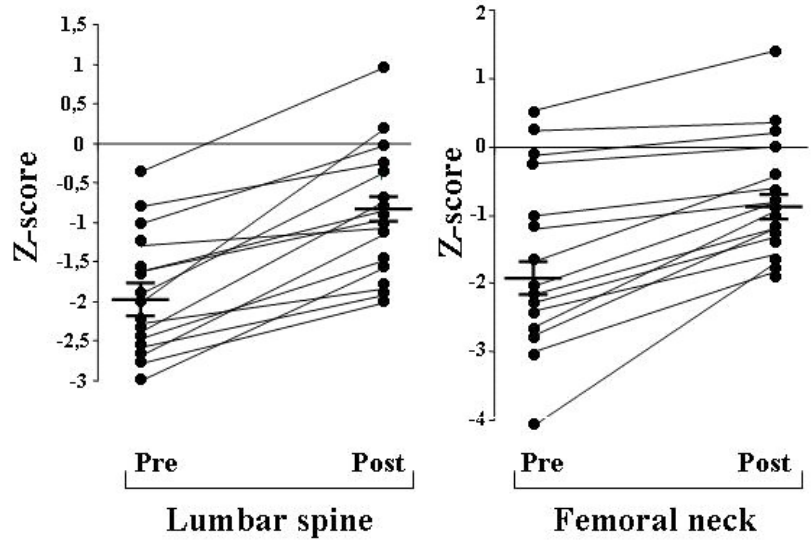


Figure 2

Table 2. Results of the longitudinal evaluation in 32 long-term survivors after allogeneic SCT, before entering the trial with zoledronic acid

Variable	**First evaluation**	**Second evaluation**	**Controls** (n=64)
Gender (F/M)	15/17	15/17	28/32
BMI (kg/m^2)	25.5±2.3	25.3±2.3	24.8±3.0
No. of patients continuing corticosteroids/ dose (mg/day)	8/17.5±15.1	4/12.3±10	-
Women on HRT (n/total women)	8/15	8/15	-
Calcium (mmol/l)	2.37±0.12	2.38±0.11	2.36±0.12
Phosphorus (mmol/l)	1.07±0.16	1.06±0.2	1.0±0.18
Bone alkaline phosphatase (mcg/l)	10.2±5.3	12.1±5.8	15.2±4.1
Creatinine (μmol/l)	86±8.5	87±8	80±10
Albumin (g/dl)	4.2±0.4	4.2±0.5	4.3± 0.3
Osteocalcin (ng/ml)	12±4.4*	12.7±5.3*	15.8± 2.5
iPTH (ng/l)	40±10	43±9	36.9± 10.6
Urinary hydroxyproline excretion ($\mu mol/l/m^2$)	135±31*	129.6±28*	115±15
Urinary hydroxyproline/creatinine	9.2	8.8	8.6
Daily calcium excretion (mmol/24h)	3.5±1.5	3.3±1.3	3.35±1.1
BMD at the lumbar spine (g/cm^2)	0.96±0.13*	0.93±0.15**	1.04±0.09
% change from baseline		-3.42±7%	
BMD at the femoral neck (g/cm^2)	0.83±0.1*	0.80±0.13**	0.92±0.11
% change from baseline		-3.8±8.1%	

Values are expressed as means ± SD. BMI: body mass index; iPTH: intact parathyroid hormone; BMD: bone mineral density. The second evaluation was performed at an interval of 8-18 months (median, 12.2months), and during calcium (500 mg daily) and vitamin D (400 IU daily) supplementation. Previous corticosteroid and cyclosporin A cumulative dose was 5.8±4 g and 64±12 g given for 248±190 and 338±180 days, respectively. Normal ranges: serum calcium: 2.2-2.6 mmol/L; serum phosphorus: 0.7-1.35 mmol/L; bone alkaline phosphatase: 7-25 μg/L; creatinine: <133 μM/L; albumin: 3.6-5.2 gr/dL; intact osteocalcin: 2-22 ng/ml; iPTH: 10-75 ng/L; urinary hydroxyproline excretion: 60-190 (μmol/m2); Ca excretion: 1.3-6.5 mmol/24h. *: $p<0.05$; ** $p<0.01$ vs. controls.

The percentage and absolute increase in FN BMD were inversely correlated with pre-treatment values at the same skeletal site (r= -0.778; P =0.014 and r=-0.79; P=0.01, respectively), indicating greater improvement in patients with lower initial bone density. On the other hand, in the untreated group of 15 patients, no significant change occurred in bone turnover markers (**Table 3**) and bone density at both LS (-2.1 %) and FN (-2.3%) (P=NS). The change in BMD between the treated and untreated groups was significant at both LS and FN (P<0.00001), as was the difference in absolute BMD values after the 12 month treatment (P<0.001 and P<0.05 for LS and FN, respectively) (**Table 3**).

Adverse effects of zoledronic acid

Side effects, including flu-like symptoms, myalgia and nausea, occurred in 12 patients (80%); they were generally mild and well controlled using 15 mg prednisone as pre-medication, one hour before zoledronic acid administration. Kidney function was carefully monitored every month and the changes did not exceed 5% of the baseline levels over the whole observation period (**Table 3**).

Effects of zoledronic acid on marrow CFU-F cells

We have recently documented a severe and long-lasting decrease in marrow CFU-F cells after allogeneic SCT (20). In the present study, enriched mesenchymal stem cells were used to obtain CFU-F cells. At the baseline evaluation, the marrow compartment of CFU-F cells, resulted reduced 3- to 4-fold in transplanted patients compared to normal donors [mean CFU-F/10^6 cells plated ± SEM: 9.9±2.9 (range: 0-46) (n=19) *vs* 46.8±4.4 (range: 29-59) (n=15); *P*=0.000009] (**Figure 3**).

Zoledronic acid significantly improved *in vitro* growth of marrow CFU-F cells in the whole group of 12 patients (mean CFU-F/10^6 cells plated ± SEM: 8.7±1.7 *vs* 21.9±3.5 before and after treatment, respectively; *P*=0.002). However, in 7/12 patients the number of colonies in the CFU-F assay still remained below the normal range. In six patients we performed CFU-F colony assays also 3 and 6 months after the beginning of zoledronic acid treatment, and the number of their colonies progressively increased from the 3rd to the 12th month (mean CFU-F/10^6 cells plated ± SEM: 12.1±3, 16 ±8 and 23.2±4 at 3, 6 and 12 months after zoledronic acid introduction, respectively), suggesting a continuous positive effect

of zoledronic acid on CFU-F colonies up to nine months after the withdrawal of treatment. These CFU-F cells clearly showed osteoblastic differentiation as documented by positivity to ALP staining after their replating in the osteoinductive conditions described in the Method section. On the contrary, only a marginal increase in the CFU-F cells was detected in 10 untreated patients who were evaluated after an interval of time similar to that of the zoledronic acid-treated cohort (12±2 and 11±2 months in the untreated and treated cohorts, respectively): mean CFU-F/10^6 cells plated ± SEM: 7.1±1.6 *vs* 8.9±2.0, respectively; P =0.48 (**Figure 3**). The difference in CFU-F cells at the second evaluation between treated and untreated patients was highly significant (P<0.00001).

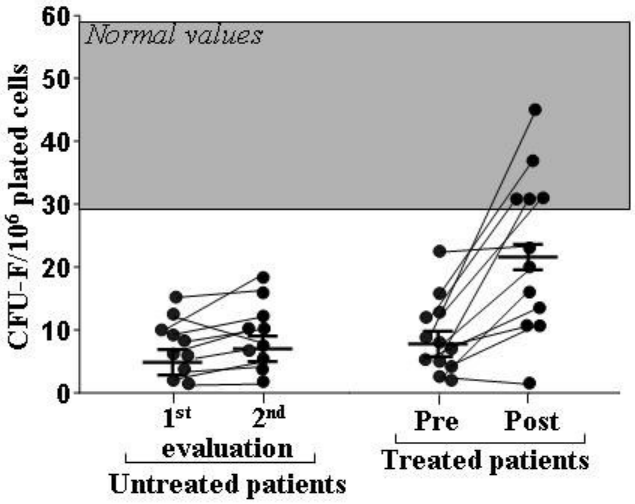


Figure 3

Figure 3. Clonogenic fibroblast progenitors in transplanted patients. Bone marrow enriched mesenchymal cells were used for colony forming unit-fibroblast (CFU-F) assay. CFU-F assay was repeated after 12±2 months from the beginning of zoledronic acid treatment in 12 patients and after 11±4 months in 10 untreated patients. A significant increase in CFU-F cells was found in the treatment group, while only a marginal increase was shown in a single patient in the untreated group. Shaded area: range values obtained in 15 normal individuals (bone marrow donors). Horizontal bars represent mean values, vertical bars represent the standard error of mean.

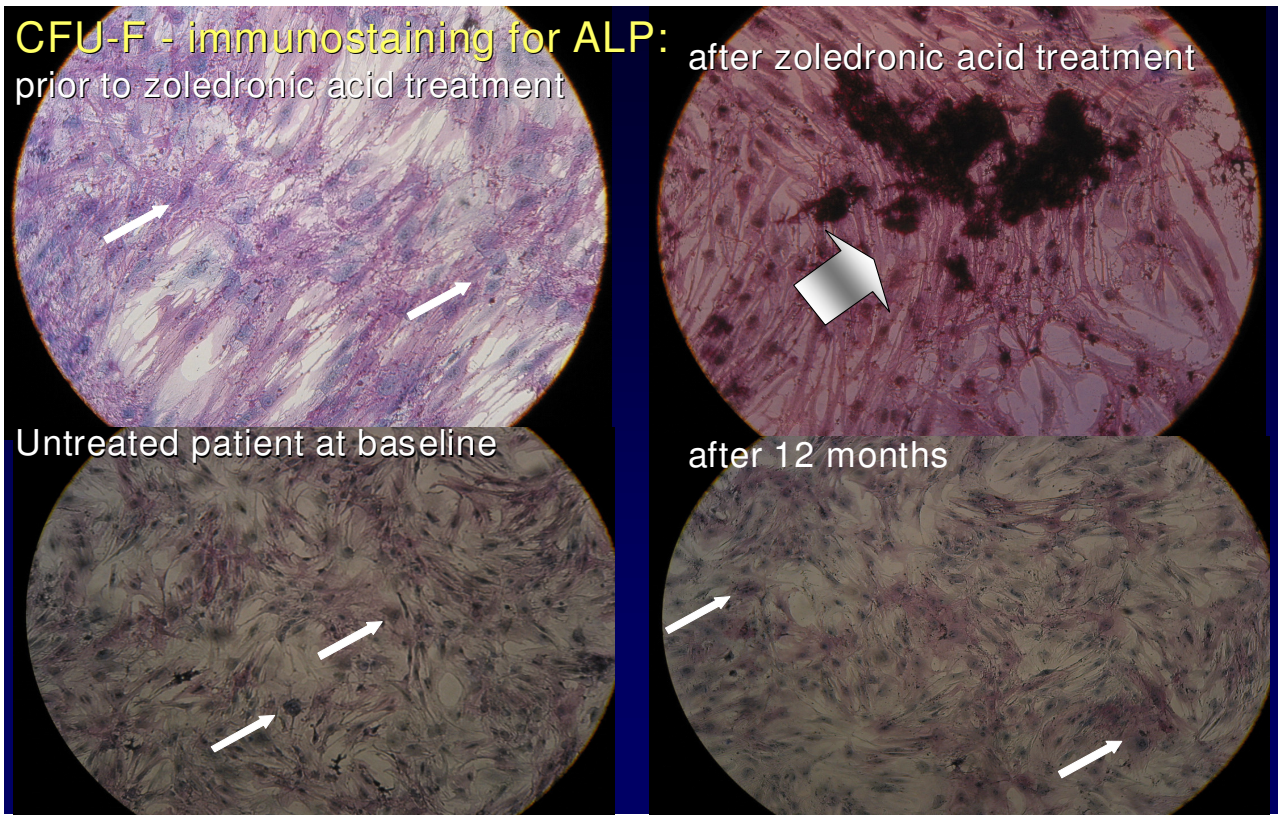


Figure 4. Immunostaining for ALP at baseline and after 12 months in a patient treated with zoledronic acid and in an untreated patient.

Discussion

In this longitudinal study, we have confirmed our previous finding on persistent bone loss up to 3 years after allo-SCT (20,21). Age, time since SCT, hypogonadism, presence of cGHVD and its steroid treatment were all related in some way to bone loss; however, one of the most important roles in persistent bone loss seems to be played by the transplant procedure itself, possibly through persistently reduced regenerative capacity of osteoblastic precursors (20,21,24,25). Femoral BMD was more strongly influenced by BMI than the lumbar one, while the latter was related to amenorrhea duration and corticosteroid exposure. The major exposure to glucocorticoids and other immunosuppressive treatments in allo-SCT recipients occurs early after transplant for the prevention and treatment of GVHD. Since the repopulating capacity of osteoblast precursors has also been proven to be related to cGVHD (20); patients affected by this complication seem to have multiple risk factors that predispose them to continuous bone loss (26). Moreover, all male patients on glucocorticoid treatment presented inhibited gonadal axis with consequently transient but likely metabolically relevant hypogonadism.

Table 3. Characteristics of patients treated with zoledronic acid *vs.* non treated group. Both groups were receiving calcium and vitamin D supplementation since the first evaluation

	Treated with zoledronic acid (n=15)		**Non treated group (n=15)**	
	Pre-treatment	After 12-month	Baseline	After 12 months
Gender (F/M)	7/8	7/8	7/8	7/8
BMI (kg/m^2)	25.4±2.3	25.3±2.5	25.75±2.25	25.6±2.6
No. of patients on corticosteroids/ dose taken (g/m^2)	*5/7.2±5.5*	4/8.1±5.8	4/6.9±5.5	3/7.4±5.7
Women on HRT (no./total women)	4/7	4/7	4/7	4/7
Calcium (mmol/l)	2.38±0.11	2.35±0.11	2.38±0.12	2.37±0.12
Phosphorus (mmol/l)	1.06±0.2	1.05±0.2	1.07±0.15	1.06±0.2
Bone alkaline phosphatase (mcg/l)	11.2±4.8	15.2±5.6*	13.3±4.9	12±5.3
Creatinine (µmol/l)	87±8	91±9	87.5±9.5	87±9
Albumin (g/dl)	4.2±0.5	4.3±0.4	4.2±0.4	4.2±0.4
Osteocalcin (ng/ml)	11±7.2	16±6.3	13.3±4.2	13.7±4.1
Parathyroid hormone (ng/l)	41±9	38.5±10	38.3±10.5	40±10
Urinary hydroxyproline excretion (µmol/l/m^2)	129.6±28	76±25**	130±34	126±33
Urinary hydroxyproline/creatinine	*8.8*	*8.5*	*9.0*	8.9
Urinary calcium excretion (mmol/24h)	3.4±1.5	3.2±1.4	3.18±1.6	3.19±1.6
BMD at lumbar spine (g/cm^2)	0.89±0.1	0.98±0.1**	0.93±0.12	0.89±0.03[a a]
% change		**9.8±7%**		**-2.1±*3*%** [a aa]
BMD at femoral neck (g/cm^2)	0.74±0.1	0.82±0.1*	0.78±0.09	0.76±0.04 [a]
% change		**6.47±7%**		**-2.3±*3.5*%** [a aa]

Zoledronic acid was administered at a dose of 4 mg intravenously every 28 days for three consecutive months. Values are expressed as mean ± SD. Abbreviations: BMI: body mass index; HRT: hormone replacement therapy; BMD: bone mineral density. For normal ranges see legend to Table 2; *: $p<0.005$; **: $p<0.001$ *vs.* baseline; [a] :$p<0.05$, [a] [a]:$p<0.01$ and [a aa] *:$p<0.0001$ vs*. treated group.

The condition of hypogonadism and cGVHD largely overlapped in the whole population. Thus, it is difficult to evaluate the effects of these two factors separately, especially in men, where hypogonadism has a shorter duration and is milder than in women. Since the liver is one of the most frequent targets of cGVHD, an increase in liver enzymes often does not permit the introduction of replacement therapy for hypogonadism. Furthermore, currently there is a recommendation to not treat mild transient male hypogonadism.

Stern *et al.* specifically evaluated the effects of cGVHD on bone mass over a 9 month period (27). An important average bone loss was shown in 9 patients, (decrease, -9.5%) and was characterized by great individual variability (range, +0.85% to -21.55%). Surprisingly, while the initial amount of bone loss was related to cGVHD presence in our study, the relationship was lost at the longitudinal evaluation. After the initial observation of a relationship between BMD reduction and glucocorticoid exposure (20,21), we started to treat cGVHD with the new immunosuppressive agent mycophenolate mofetil, known to cause less bone loss (28,29). The most plausible hypothesis on the loss of the relationship between BMD values and GVHD consists of variable bone effects of different immunosuppressive regimens. Other factors can be represented by a relatively short observation period (12 months) for an unsevere GVHD. The only correlation at the limit of significance was found between the amount of FN bone loss and CsA treatment duration.

Most of the longitudinal studies investigated bone loss during the first year after SCT, comparing post-transplant BMD with pre-transplant values. The sharpest bone loss at trabecular and cortical sites occured within the first 6-12 months after allo-SCT. In our study, bone loss was evaluated comparing two BMD measurements performed after the transplant. The baseline evaluation that was done in the later stage after SCT can explain the lower rate of subsequent bone loss found in this study.

Considering previous data on the ineffectiveness of common intervention in preventing bone loss (6), transplanted patients need to be treated by highly potent, specific agents. Our study aimed to investigate the bone effects of intravenous zoledronic acid given as three monthly doses of 4 mg each in patients who had undergone allogeneic SCT. This produced beneficial effects on lumbar and femoral BMD, largely higher than the one obtained by an oral bisphosphonate (30). Indeed, in our previous study a treatment with risedronate for 12 months increased BMD at the LS by 5.9% and prevented bone loss at the femoral neck (30). Zoledronic acid improved bone density also at cortical rich bone (11).

In this study, zoledronic acid was given as three consecutive doses of 4 mg each because of the evidence of multifactorial, conspicuous and persistent bone loss in long–term

survivors after allo-SCT. With this schedule we had tried to obtain rapid and measurable effects on bone mass. Given the persistent bone loss at trabecular and cortical rich skeletal sites in transplant recipients, zoledronic acid may be considered the treatment of choice in the near-transplant period for the prevention of bone loss. Moreover, given as intermittent short-lasting administration, this agent can be easily added to the complex therapeutic regimens generally administered to transplanted patients. Several infusion protocols have been used for the treatment of osteoporosis, but no standard regimen is available. In the study by Reid et al., a total dose of 4 mg of zoledronic acid was administered during 1 year with different schedules to menopausal women with low BMD. This caused a 4.3 to 5.1% and a 2.7 to 3.1% increase in LS and FN BMD, respectively, and was associated with a significant suppression of bone turnover markers (16). Another regimen was used in a multicenter study by Smith et al., who investigated the effects of 4 mg of zoledronic acid repeated at 3-month intervals for one year in men with non-metastatic prostate cancer during androgen deprivation therapy; a 5.6% increase in lumbar BMD was observed (11).

Side effects were generally mild and well controlled by low-dose steroid pre-medication. No alteration in kidney function was detected at any time point during the study, and liver enzymes did not worsen during the treatment period. As a matter of fact, zoledronic acid has proven to have a lower nephrotoxic potential than pamidronate in rat models (31).

No conclusion can be made about fracture risk because this study is not sufficiently powerful to evaluate fracture incidence as a clinical end-point. Whether any treatment is able to reduce fracture risk after transplant still remains to be determined.

Beneficial effects on lumbar and femoral BMD of zoledronic acid treatment in our transplanted patients was associated with a significant improvement of the clonogenic fibroblast progenitors belonging to the osteogenic stromal lineage. Although the specific mechanisms whereby bisphosphonates exert their beneficial osteotropic effect are not yet completely clarified, it is generally accepted that they have a direct effect on osteoclasts, inducing their apoptosis (32-34), inhibiting their bone-resorbing activity (35) and blocking their precursor proliferation (36-38). This bisphosphonate-mediated anti-resorptive action has been reported to be related to the inhibition of the osteoclastic mevalonate pathway (34). In addition, recent *in vitro* findings suggest that bisphosphonates have multiple effects on bone turnover, in fact, they may directly enhance osteoblast proliferation and differentiation (39,40), prevent osteoblast apoptosis (41) and regulate osteoblast production of extracellular matrix proteins (42). All of these mechanisms further contribute to the inhibitory effect on bone turnover. Moreover, there is *in vitro* evidence that bisphosphonates increase osteoblastic

production of osteoprotegerin, which is needed to neutralize the receptor activator of the nuclear factor-kB ligand (RANKL) essential for osteoclast formation and activation (43).

Viereck *et al*. have shown that *in vitro* pretreatment with pamidronate or zoledronate prevents the inhibitory effects of dexamethasone on osteoprotegerin production in the bone environment, suggesting that this mechanism may contribute to the bisphosphonate-induced enhancement of osteoclastic apoptosis in glucocorticoid-induced bone loss (43). The positive effects on osteoblasts may be particularly important in allo-transplanted patients, since previous evidence indicated a persistently reduced regenerative and functional capacity of osteoblast precursors (20,26). Bisphosphonates were also demonstrated to stimulate *in vitro* formation of human and murine osteoblast precursors and of mineralized nodules, the latter probably through secretion of basic fibroblast growth factor stimulating CFU-F proliferation (42,44). Our *in vitro* results are in line with these studies. We have used CFU-F, as they are well recognized as the initial cell source of early and late osteoblast precursors (45). A significant improvement of *in vitro* growth of osteogenic progenitors was induced by *in vivo* zoledronic acid administration. In untreated patients, the number of CFU-F colonies marginally improved, likely due to the time elapsed from the effects of cytotoxic drugs and transplant-related cytokine storm. The difference between spontaneous and zoledronate-induced improvement was highly significant. In agreement with a larger study (46), we documented a recipient origin of clonogenic fibroblast progenitors also after zoledronic acid treatment (Selleri, unpublished data). The increase in BMD and clonogenic fibroblast progenitors was accompanied by a significant decrease in hydroxyproline excretion and by an increase in serum bone alkaline phosphatase, while osteocalcin increase was just below the limit of statistical significance. The increase in marrow osteogenic precursors and bone alkaline phosphatase without significant increase in serum osteocalcin, could be explained by a strong "in situ" stimulatory effect of zoledronic acid on the osteoblastic compartment concentrated in local niches of the stromal microenvironment. Such an effect was unable to be detectable as changes in serum bone formation markers. Indeed, controversial results were reported in serum osteocalcin levels during bisphosponate treatment for post-transplant osteoporosis (47-49).

In conclusion, persistent multifactorial bone loss has been confirmed in patients up to 36 months after allo-SCT. Most important risk factors included persistently reduced regenerating capacity of normal osteogenic cell compartment, ovarian failure and the presence of cGVHD, requiring long-term immunosuppressive treatments. Zoledronic acid was easily manageable and effective in increasing densitometric values at both trabecular and

cortical skeletal sites. At least part of this effect can be ascribed to the ability in reversing the persistent post-transplant reduction of the clonogenic fibroblast progenitors belonging to the osteogenic stromal lineage. As intravenous bisphosphonates represent the most potent treatment option for post-transplant osteoporosis currently available, transplanted patients who are at high risk for fast and/or persistent bone loss should receive preventive treatment. Studies evaluating its preventive use should be carried out. Currently, the choice of oral/intravenous bisphosphonate administration in patients who had undergone allogeneic SCT should be made on the basis of individual clinical conditions, including the presence, grading and localization of GVHD, and prevalent site of bone loss.

References

1. Stern JM, Sullivan KM, Ott SM, Seidel K, Fink JC, Longton G, Sherrard DJ. Bone density loss after allogeneic hematopoietic stem cell transplantation: a prospective study. *Biol Blood Marrow Transplant* 2001; 7: 257-264.
2. Gandhi MK, Lekamwasam S, Inman I, Kaptoge S, Sizer L, Love S, Bearcroft PW, Milligan TP, Price CP, Marcus RE, Compston JE. Significant and persistent loss of bone mineral density in the femoral neck after haematopoietic stem cell transplantation: long-term follow-up of a prospective study. *Br J Haematol* 2003; 121: 462-468.
3. Weilbaecher KN. Mechanisms of osteoporosis after hematopoietic cell transplantation. *Biol Blood Marrow Transplant* 2000; 6:165-71.
4. Katz IA, Epstein S. Perspectives. Post-transplantation bone disease. *J Bone Min Res* 1992; 7:123-126.
5. Schimmer AD, Minden MD, Keating A. Osteoporosis after blood and marrow transplantation: clinical aspects. *Biol Blood Marrow Transplant* 2000; 6:175-81.
6. Valimaki MJ, Kinnunen K, Volin L, Tahtela R, Loyttyniemi E, Laitinen K, Makela P, Keto P, Ruutu T. A prospective study of bone loss and turnover after allogeneic bone marrow transplantation: effect of calcium supplementation with or without calcitonin. *Bone Marrow Transplant* 1999; 23:355-361.
7. Castelo-Branco C, Rovira M, Pons F, Duran M, Sierra J, Vives A, Balasch J, Fortuny A, Vanrell J. The effect of hormone replacement therapy on bone mass in patients with ovarian failure due to bone marrow transplantation. *Maturitas* 1996; 23:307-312.
8. Massenkeil G, Fiene C, Rosan O, Michael R, Reisinger W, Arnold R. Loss of bone mass and vitamin D deficiency after hematopoetic stem cell transplantation: standard prophylactic measures fail to prevent osteoporosis. *Leukemia* 2001; 15:1701-5.
9. Marcus R, Wong M, Heath H III, Stock JL. Antiresorptive treatment of postmenopausal osteoporosis: comparison of study designs and outcomes in large clinical trials with fracture as an endpoint. *Endocr Review* 2002; 23:16-37.
10. Delmas PD. Treatment of postmenopausal osteoporosis. *Lancet* 2002; 8:2018-26.
11. Smith MR, Eastham J, Gleason DM, Shasha D, Tchekmedyian S, Zinner N. Randomized controlled trial of zoledronic acid to prevent bone loss in men receiving androgen deprivation therapy for nonmetastatic prostate cancer. *J Urol* 2003; 169:2008-12.
12. Diez-Perez A. Bisphosphonates. Maturitas. 2002; 43:S19-26.
13. Body JJ, Lortholary A, Romieu G, Vigneron AM, Ford J. A dose-finding study of zoledronate in hypercalcemic cancer patients. *J Bone Miner Res* 1999;14:1557-61.
14. Lipton A, Small E, Saad, Gleason D, Gordon D, Smith M, Rosen L, Kowalski MO, Reitsman D, Seaman J. The new bisphosphonate, Zometa (zoledronic acid), decreases skeletal complications in both osteolytic and osteoblastic lesions: a comparison to pamidronate. *Cancer Invest* 2002; Suppl 2:45-54.

15. Boutsen Y, Jamart J, Essenlinckx W, Devogelaer JP. Primary prevention of glucocorticoid-induced osteoporosis with intravenous pamidronate and calcium: a prospective controlled 1-year study comparing a single infusion, an infusion given once every 3 months, and calcium alone. J Bone Miner Res 2001;16:104-12.

16. Reid IR, Brown JP, Burckhardt P, Horowitz Z, Richardson P, Trechsel U, Widmer A. Intravenous zoledronic acid in postmenopausal women with low bone mineral density. *N Engl J Med* 2002; 346:653-661.

17. Adami S, Bevilacqua M, Broggini M, Filipponi P, Ortolani S, Palummeri E, Ulivieri F, Nannipieri F, Braga V. Short-term intravenous therapy with neridronate in Paget" disease. *Clin Experiment Rheumatol* 2002; 20:55-58.

18. Aparicio A. Gardner A, Tu Y, Savage A, Berenson J, Lichtenstein A. In vitro cytoreductive effects on multiple myeloma cells induced by bisphosphonates. *Leukemia* 1998;12:220-229.

19. Coukell AJ, Markham A. Pamidronate. A review of its use in the management of osteolytic bone metastases, tumour-induced hypercalcaemia and Paget's disease of bone. *Drugs Aging*. 1998;12:149-68.

20. Tauchmanovà L, Serio B, Del Puente A, Risitano AM, Esposito A, De Rosa G, Lombardi G, Colao A, Rotoli B, Selleri C. Long-lasting bone damage detected by dual energy x-ray absorptiometry, phalangeal osteosonogrammetry and *in vitro* growth of marrow stromal cells after allogeneic stem cell transplantation. *J Clin Endocrinol Metab* 2002; 87: 5058-5065.

21. Tauchmanovà L, De Rosa G, Serio B, Fazioli F, Mainolfi C, Lombardi G, Colao A, Salvatore M, Rotoli B, Selleri C. Avascular necrosis in long-term survivors after allogeneic or autologous stem cell transplantation: a single center experience and a review. *Cancer* 2003; 97:2453-2461.

22. World Health Organization Study Group. Assessment of fracture risk and its application to screening for postmenopausal osteoporosis. WHO Technical Report Series 843. 1994; WHO, Geneve.

23. del Puente A, Heyse SP, Mandes MG, Mantova D, Carpinelli A, Nutile G, Oriente P. Epidemiology of osteoporosis in women in southern Italy. *Aging Clin Exp Res* 1998; 10: 53-58.

24. Lee WY, Cho SW, Oh ES, Oh KW, Lee JM, Yoon KH, Kang MI, Cha BY, Lee KW, Son HY, Kang SK, Kim CC 2002 The effect of bone marrow transplantation on the osteoblastic differentiation of human bone marrow stromal cells. *J Clin Endocrinol Metab* 2002; 87: 329-335.

25. Banfi A, Podesta M, Fazzuoli L, Sertoli MR, Venturini M, Santini G, Cancedda R, Quarto R. High-dose chemotherapy shows a dose-dependent toxicity to bone marrow osteoprogenitors: a mechanism for post-bone marrow transplantation osteopenia. *Cancer* 2001; 92:2419-28.

26. Schulte CMS, Beelen DW. Bone loss following hematopoietic stem cell transplantation: a long-term follow-up. *Blood* 2004; 103:3635-43.

27. Stern JM, Chesnut CH III, Bruemmer B, Sullivan KM, Lenssen PS, Aker SN, Sanders J. Bone density during treatment of chronic GVHD. *Bone Marrow Transplant* 1996; 17:395-400.

28. Aalamian Z. Reducing adverse effects of immunosuppressive agents in kidney transplant recipients. *Prog Transplant*. 2001;11:271-82.

29. Lo A, Alloway RR. Strategies to reduce toxicities and improve outcomes in renal transplant recipients. *Pharmacotherapy.* 2002; 22:316-28.

30. Tauchmanovà L, Selleri C, Esposito M, Di Somma C, Orio F Jr, Bifulco G, Palomba S, Lombardi G, Rotoli B, Colao A. Beneficial treatment with risedronate in long-term survivors after allogeneic stem cell transplantation for hematological malignancies. *Osteoporosis International*, 2003, 14:1013-1019.

31. Green JR, Seltenmeyer Y, Jaeggi KA. Renal tolerability profile of novel, potent bisphosphonates in two short-term rat models. *Pharmacol Toxicol* 1997; 80: 225-230.

32. Rogers M J, Chilton KM, Coxon FP, Lawry J, Smith MO, Suri S, Russell RG. Bisphosphonates induce apoptosis in mouse macrophage-like cells in vitro by a nitric oxide-independent mechanism. J Bone Miner Res 1996; 11: 1482-1491.

33. Ito M, Amizuka N, Nakajima T, Ozawa H. Ultrastructural and cytochemical studies on cell death of osteoclasts induced by bisphosphonate treatment. *Bone* 1999; 25: 447-452.

34. Reszka AA, Halasy-Nagy JM, Masarachia P J, Rodan GA. Bisphosphonates act directly on the osteoclast to induce caspase cleavage of Mst1 kinase during apoptosis. *J Biol Chem* 1999; 274: 34967-34973.

35. Selander K, Lehenkari P, Väänänen HK. The effects of bisphosphonates on the resorption cycle of isolated osteoclasts. *Calcif Tissue Int* 1994; 55:368-375, 1994.

36. Löwik CWGM, van der Pluijm G, van der Wee-Pals LJA, van Treslong-De Groot HB, Bijvoet OLM. Migration and phenotypic transformation of osteoclast precursors into mature osteoclasts: the effect of a bisphosphonate. *J Bone Miner Res* 1988; 3*:* 185-1992.

37. Hughes DE, MacDonald BR, Russell RGG, Gowen M. Inhibition of osteoclast-like cell formation by bisphosphonates in long-term cultures of human bone marrow. *J Clin Invest* 1989; 83*:* 1930-1935.

38. Solomon CG. Bisphosphonates and osteoporosis. *N Engl J Med* 2002; 346: 642.

39. Reinholz GG, Getz B, Pederson L, Sanders ES, Subramaniam M, Ingle JN, Spelsberg TC. Bisphosphonates directly regulate cell proliferation, differentiation, and gene expression in human osteoblasts. *Cancer Res.* 2000; 60:6001-6007.

40. Fromigue O & Body JJ. Bisphosphonates influence the proliferation and the maturation of normal human osteoblasts. *J Endocrinol Invest* 2002; 25:539-546.

41. Plotkin LI, Weinstein RS, Parfitt AM, Roberson PK, Manolagas SC, Bellido T. Prevention of osteocyte and osteoblast apoptosis by bisphosphonates and calcitonin. *J Clin Invest* 1999; 104: 1363-1374.

42. Giuliani N, Pedrazzoni M, Negri G, Passeri G, Impicciatore M, Girasole G. Bisphosphonates stimulate formation of osteoblast precursors and mineralized nodules in murine and human bone marrow cultures in vitro and promote early osteoblastogenesis in young and aged mice in vivo. *Bone* 1998; 22: 455-461.

43. Viereck V, Emons G, Lauck V, Frosch KH, Blasche S, Grundker C, Hofbauer LC. Bisphosphonates pamidronate and zoledronic acid stimulate osteoprotegerin production by primary human osteoblasts. *Biochem Biophys Res Commun* 2002; 291: 680-686.

44. Im GI, Qureshi SA, Kenney J, Rubash HE, Shanbhag AS. Osteoblast proliferation and maturation by bisphosphonates. *Biomaterials* 2004; 25: 4105-4115.

45. Pittenger MF, Mackay AM, Beck SC, Jaiswl RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal cells. *Science* 1999; 284 :143-147.

46. Galotto M, Berisso G, Delfino L, Podestà M, Ottagio L, Dallorso S, Dufour C, Ferrara GB, Abbondandolo A, Dini G, Bacigalupo A, Cancedda R, Quarto R. Stromal damage as consequence of high dose chemo/radiotherapy in bone marrow transplant recipients. *Exp Hematol* 1999; 27, 1460-1466.

47. Bianda T, Linka A, Junga J, Brunner H, Steinert H, Kioski W, Schmid C. Prevention of osteoporosis in heart transplant recipients: a comparison of calcitriol with calcitonin and pamidronate. *Calcif Tissue Int* 2000; 67: 116-121.

48. Cruz DN, Brickel HM, Wysolmerski JJ, Gundberg CG, Simpson CA, Kliger AS, Lorber MI, Basadonna GP, Friedman AL, Insogna KL, Bia MJ. Treatment of osteoporosis and osteopenia in long-term renal transplant patients with alendronate. *Am J Transplant* 2002; 2: 62-67.

49. Haas M, Leko-Mohr Z, Roschger P, Kletzmayr J, Schwarz C, Mitterbauer C, Steininger R, Grampp S, Klaushofer K, Delling G, Oberbauer R. Zoledronic acid to prevent bone loss in the first 6 months after renal transplantation. *Kidney Int* 2003; 63:1130-1136.

Effects of various anti-reabsorptive treatments on bone mineral density in hypogonadal young women after allogeneic stem cell transplantation

Introduction

Bone loss consequent to allogeneic stem cell transplantation (allo-SCT) has been described by many authors (1-6) as being more persistent and severe at corticacal bone (femoral neck) when compared to trabecular sites (lumbar spine) (4,7). Damaging effects on bone mass were attributed to hypogonadism, immunosuppressive and myeloablative treatments, immobilization, increased cytokine release, reduced dietary intake and metabolic alterations of calcium and vitamin D (5,6). One of the most important risk factors for bone loss is represented by precocious ovarian failure (POF), which is also the most common complication of allo-SCT (8-10). Calcium and vitamin D supplementation and hypogonadism replacement are commonly used in clinical practice after SCT, while bisphosphonates are added only in patients with osteoporosis. Little data is available so far on the effects of preventive or therapeutic intervention on post-transplant bone loss (1,11-13). To the best of our knowledge, no systematic evaluation has been performed to compare the effects of different agents for prevention or treatment and of osteoporosis consequent to allo-SCT. All commonly employed antireabsorptive treatments have been shown to improve bone mineral density (BMD) and decrease the rate of fractures in post-menopausal and glucocorticoid-induced osteoporosis (14-17). However, the use of these treatments in transplanted patients may be limited by the general health conditions and by the simultaneous presence of other complications, including acute and chronic graft-versus-host disease (a- and cGVHD). Liver, skin and gastrointestinal tract are the most common localization of cGVHD (18) and in the active phase may represent a contraindication for the use of oral or trans-dermal therapies; even a mild chronic GVHD may reduce gastrointestinal or dermal drug absorption. Furthermore, allo-SCT patients usually run into long-lasting and complex therapeutic regimens in order to prevent or treat other complications; thus, the management of osteoporosis can be difficult in some of them.

The present study was aimed at investigating the 12-month effects of various antiresorptive treatments on lumbar and femoral BMD and bone turnover markers in female

patients with ovarian failure and low BMD following allo-SCT for hematological malignancies.

Patients and methods

Patients and study design

Sixty women who had undergone allogeneic SCT at our Institution were enrolled in this study. Persistent amenorrhea occurred in all of them, as a consequence of chemotherapy. Precocious ovarian failure (POF) was diagnosed when gonadotropin levels were in the menopausal range and estradiol below the normal range of fertile women.

All women were evaluated for BMD at the lumbar spine (LS) and femoral neck (FN), body mass index (BMI, kg/m^2), serum osteocalcin and urinary hydroxyproline excretion, after a median period of 8 months (range, 3-17) since the transplant. Endocrine parameters regarding the reproductive function were also investigated. After the initial evaluation, the patients were divided into four groups of 15 women each. All the patients received calcium 1000 mg and vitamin D 800 IU supplementation that was administered orally once daily, between 07:00 p.m. and 08:00 p.m. Group 1 received only calcium and vitamin D during the whole observational period. In group 2, estradiol 2 mg daily and dihydroprogesterone 10 mg for 14 days a month were added for 12 consecutive cycles. Group 3 was treated with risedronate (35 mg weekly, orally) for 12 months and group 4 by zoledronic acid at the dose of 4 mg i.v. every 28 days for 3 consecutive months. The four groups were all similar in age, BMI, underlying disease, time elapsed from transplant, amenorrhea duration and cGVHD features (**Table 1**).

The determination of densitometry and bone turnover markers was repeated 12 months after the first evaluation. None of the participants had been given previous treatments for osteoporosis. Written informed consent was obtained from all of the patients in accordance with the institutional guidelines; the study was designed in accordance with the Helsinki II Declaration on treatment of human subjects.

Transplantation procedure

All patients had been successfully allo-transplanted with unmanipulated marrow from a HLA identical sibling. Leukemia was the most common indication for allo-SCT. All patients had undergone conditioning with the BU-CY2 regimen (busulphan 16 mg/kg, cyclophosphamide 120 mg/kg) and had received cyclosporine A (CsA) (1 mg/kg/day by continuous i.v. infusion from day -1 to day + 20 and then 8 mg/kg/day orally) plus short-course methotrexate (10 mg/m^2 on

days +1,+3 and +6) as prophylaxis for GVHD. No patient had received radiation therapy. Patients with a- and cGVHD had been treated by methylprednisone (2 mg/kg/day or 1 mg/kg/day for 15 days in patients with aGVHD and cGVHD, respectively) and CsA (5 mg/kg/day) before the first evaluation of bone status. During the study, 53% of patients developed or had persistent cGVHD requiring immunosuppressive therapy as above described. As a significant correlation was found between corticosteroid treatment and bone damage at the initial evaluation, steroids were avoided whenever possible thereafter. During the study period, cGVHD was treated by CsA + mycophenolate mofetil at a dose of 30 mg/kg/day; low-dose corticosteroids were continued in addition to these immunosuppressants in 11 patients only. The median cumulative dose of steroids given to this cohort of patients prior to study entry was equivalent to 6.4 g of prednisone (range 0.9-20) for 3-36 months, while the average dose administered during the study was 1.3 ± 0.9 g for 3-10 months. The number of patients treated by steroids and the dose used during the study was similar among the four groups.

Gonadal status assessment

At study entry, all of the women underwent measurement of serum FSH, LH, 17-β-estradiol, testosterone and dehydroepiandrosterone-sulphate (DHEA-S) in a single sample taken at 08:00 a.m. The same parameters were again evaluated after six and 12 months. All measurements were performed by commercially available kits: FSH and LH with radioimmunoassay (RIA, Biodata, Rimini, Italy), testosterone, DHEA-S and estradiol using Immulite, solid phase chemoluminescent enzyme immunoassay (DPC, Los Angeles, CA, USA).

Bone metabolism assessment

Serum calcium, phosphorus, creatinine, bone alkaline phosphatase (ALP), intact parathyroid hormone (iPTH) and osteocalcin were determined in blood samples drawn at initial evaluation and after 6 and 12 months. Blood samples were collected in the morning after a 12-h fast. Urinary calcium, phosphorus and hydroxyproline were assayed on a 24-h urinary collection. Standard dietary restrictions were observed by all the individuals tested, starting five days prior to urinary collection. Intact PTH and serum osteocalcin levels were measured by RIA (Nichols Institute Diagnostics, CA), the detection limit of the latter being 0.35 mg/L. Bone alkaline phosphatase was assayed by a direct immunoradiometric assay (Ostease, Hybritech, San Diego, CA). Hydroxyproline excretion was measured by high pressure liquid chromatography. Blood chemistry profile, including Ca, P, ALP, urinary Ca excretion and creatinine levels, were analyzed using a standard autoanalyzer.

Table 1. Clinical features of 60 transplanted female patients – women long-term survivors after allogeneic SCT

Variable	Group 1	Group 2	Group 3	Group 4
N° of patients	15	15	15	15
Age (yrs)	26.1 ± 9	26.8 ± 8.6	24.8 ± 7.8	27 ± 9
BMI (kg/m^2)	23.5 ± 2.3	23.8 ± 2.4	24.1 ± 3.0	23.2 ± 2.6
Underlying disease:				
AML in 1st CR/ CML in chronic phase	8/5/2/-/-	7/6/1/-/1	7/6/1/1/-	7/6/1/1/-
ALL in 2nd CR/ HD/ SAA				
Amenorrhea duration (months)	10.3 ±10	9 ± 9.2	10.8 ± 11	9.5 ± 10.2
Acute GVHD	6	6	7	8
Grade I-II/III-IV	5/1	4/2	5/2	6/2
Chronic GVHD	7	8	8	9
Limited/extensive form	4/3	4/4	3/5	3/6
Corticosteroid treatment#				
Duration (days)	216 ± 160	198 ± 143	247 ± 178	255 ± 150
Cumulative dose (g)*	6.9 ± 5	7.0 ± 5.4	7.7 ± 4.9	8.2 ± 4.5
Cyclosporin A treatment#				
Duration (days)	339 ± 176	351 ± 165	328 ± 140	349 ± 170
Cumulative dose (g)	61 ± 13	58 ± 14	64 ± 12	67 ± 15

Values are expressed as mean ± SD, when appropriate. Abbreviations: SCT: stem cell transplant, BMI: body mass index, AML: acute myeloid leukemia, CML: chronic myeloid leukemia, ALL: acute lymphoblastic leukemia, HD: Hodgkin's disease, SAA: severe aplastic anemia, CR: complete remission, GVHD: graft versus host disease; Symbols: #: immunosuppressive treatments before study enrollment;*: cumulative dose of corticosteroids is expressed as prednisone equivalent.

Bone density and fracture assessment

Bone density was determined by dual-energy x-ray absorptiometry (DEXA) at the lumbar spine (L1-L4) and femoral neck (FN), using Hologic QDR 1000 densitometer (Hologic, Inc., Waltham, MA). Individual BMD values were expressed as g/cm^2 and T, Z-scores. Quality control was performed by daily scanning of an anthropomorphic spine phantom. The coefficient of variation was 1.8% for the LS and 2.2% for the FN. The reference population adopted in this study was the international pooled sample provided by the manufacturer; their data, however, did not differ significantly from those obtained on a local sample in a study performed when the device was set up (19).

Standard spinal radiographs in anterior-posterior and lateral positions of vertebrae Th4 - L4 was performed in all patients at baseline and after 12 months. At baseline, two women from group 3 and three from group 4 had prevalent fractures, determined as a 20% difference in antero-posterior, middle-posterior or posterior-posterior adjacent ratio (20).

Statistical analysis

Data are reported as mean ± SD unless otherwise specified. The normal data distribution was tested by Wilk-Shapiro's test. Statistical analysis was performed by the Student's *t*-test for paired and unpaired data, as appropriate, for comparison between groups treated by different regimens. At pre-treatment evaluation, the association between BMD values, immunosuppressive treatment duration and cumulative doses, and amenorrhea period were assessed by linear regression. Comparison of the variables within the same group was performed by the Friedman test. The significance was set at 5%.

Results

Clinical features and gonadal status

Clinical features at study entry, underlying malignancies and details on previous treatments are summarized in Table 2. Women who did not undergo HRT did not experienced recovery of spontaneous menstrual cycles during the observational period. None of the women from group 2 reported menstrual cycles within 60 days after HRT withdrawal at the end of the study, their gonadotropins and estradiol being still in the postmenopausal range.

Baseline evaluation of bone turnover and BMD after SCT

Gonadotropin values were in the menopausal range, while estradiol, testosterone and DHEA-S were significantly lower than the normal range for reproductive aged women. Baseline endocrine parameters, BMD values at both skeletal sites and bone turnover markers were similar in all groups of patients (**Table 2**). According to the WHO criteria (21), osteoporosis was diagnosed in 4 patients of group 1, 5 of group 2, 5 of group 3 and 6 of group 4.

Baseline lumbar BMD correlated inversely with the duration of amenorrhea (r=-0.47; *P* =0.045). Serum osteocalcin correlated with baseline lumbar BMD (r=0.61; *P*=0.048). A borderline correlation was present between lumbar BMD and cumulative dose of corticosteroids used prior to the study entry (r=-0.49; *P*=0.05).

Treatment effects on endocrine parameters, bone turnover and BMD

Mean gonadotropin, estradiol, testosterone and DHEA-S levels did not change significantly in groups 1, 3 and 4. Gonadotropins decreased (FSH by 60%, LH by 64%) and estradiol increased (by 387%) in group 2 during HRT (P<0.0001, all), whereas testosterone and DHEA-S did not change; nevertheless, gonadotropins still remained above and estradiol below their normal ranges for reproductive aged women.

There were no significant changes in BMI, serum levels of calcium, phosphorus, albumin, calcium urinary excretion and iPTH in any group during the 12 month observation period.

When concerning with bone turnover markers, in groups 3 and 4 hydroxyproline excretion significantly decreased at 3, 6 and 12 months, when compared with baseline values (P<0.001)(**Figure 1**). Bone alkaline phosphatase and osteocalcin did not change in groups 1,2 and 3; they were found slightly but significantly increased in group 4 (P= 0.038 and P=0.032, respectively).

Two incident subclinical vertebral fractures in group 1 and one in group 2 were detected at the 12th month radiological evaluation of the spine. No non-vertebral fracture occurred during the observation period.

Table 2. Results of the initial evaluation of biochemical parameters and bone density in 60 women long-term survivors after allogeneic SCT

Variable	**Group 1** (n=15)	**Group 2** (n=15)	**Group 3** (n=15)	**Group 4** (n=15)	**Normal range**
Age (yrs)	26.1 ± 9	26.8 ± 8.6	24.8 ± 7.8	27 ± 9	
BMI (kg/m^2)	23.5 ± 2.3	23.8 ± 2.4	24.1 ± 3.0	23.2 ± 2.6	
FSH (U/L)	112 ± 51	103 ± 42	123 ± 39	115 ± 45	
LH (U/L)	62.5 ± 23	70 ± 19	58 ± 20	68 ± 18	
Estradiol (pg/ml)	21 ± 12	16 ± 14	23 ± 9	15 ± 10	
Testosterone (pg/ml)	0.35 ± 0.4	0.2 ± 0.3	0.3 ± 0.3	0.34 ± 0.5	
DHEAS (μg/dl)	130 ± 62	140 ± 67	110 ± 57	125 ± 39	
Calcium (mmol/l)	2.37 ± 0.12	2.38 ± 0.11	2.36 ± 0.12	2.37 ± 0.13	
Phosphorus (mmol/l)	1.07 ± 0.16	1.06 ± 0.2	1.1 ± 0.18	1.05 ± 0.21	
Bone alkaline phosphatase (mcg/l)	10.3 ± 5.5	12.1 ± 5.8	11.3 ± 4.1	11.2 ± 4.4	
Creatinine (μmol/l)	85 ± 8.5	87 ± 8	82 ± 10	83 ± 9	
Albumin (g/dl)	3.95 ± 0.4	3.93 ±0.38	3.9 ± 0.43	4.0 ± 0.5	
Osteocalcin (ng/ml)	12.2 ± 4.4	12.7 ± 5.3	13.3 ± 4.5	11.8 ± 3.5	
iPTH (ng/l)	34 ± 12	38 ± 9.2	39.9 ± 10.6	36.6 ± 11.3	
Urinary hydroxyproline excretion (μmol/l/m^2)	136 ± 32	129 ± 27	125 ± 25	131 ± 22	
Daily calcium excretion (mmol/24h)	3.6 ± 1.4	3.4 ± 1.4	3.35 ± 1.1	3.5 ± 1.3	
BMD at the lumbar spine (g/cm^2)	0.91 ± 0.14	0.89 ± 0.15	0.92 ± 0.1	0.9 ± 0.11	
T-score (SD)	-1.3 ± 1.3	-1.5 ± 1.2	-1.2 ± 1	-1.4 ± 1	
BMD at the femoral neck (g/cm^2)	0.77 ± 0.12	0.78 ± 0.11	0.74 ± 0.12	0.71 ± 0.13	
T-score (SD)	-1.4 ± 1.2	-1.3 ± 1.1	-1.5 ± 1.1	-1.85 ± 1.2	

Values are expressed as means ± SD. Abbreviations: BMI: body mass index; iPTH: intact parathyroid hormone; BMD: bone mineral density. Normal ranges: FSH: 2-13 U/L; LH: 2-15 U/L; 17β-estradiol: 40-250 pg/ml; testosterone: 0.2-1 pg/ml, DHEAS: 80 – 560 μg/dl; serum calcium: 2.2-2.6 mmol/L; serum phosphorus: 0.7-1.35 mmol/L; bone alkaline phosphatase: 7-25 μg/L; creatinine: <133 μM/L; albumin: 3.6-5.2 gr/dl; intact osteocalcin: 2-22 ng/ml; iPTH: 10-75 ng/L; urinary hydroxyproline excretion: 60-190 (μmol/m2); Ca excretion: 1.3-6.5 mmol/24h.

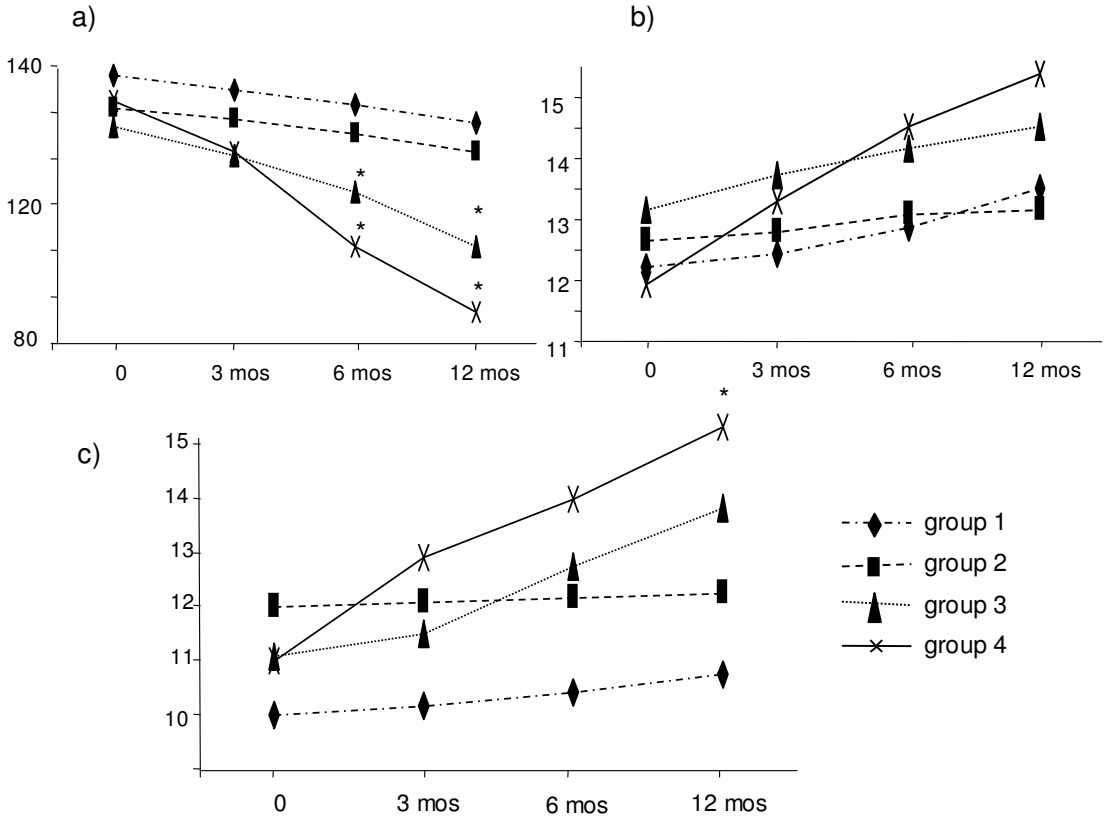


Figure 1. Bone turnover markers at months 0,3,6 and 12 of treatment. All women were treated with calcium (1000 mg daily) plus vitamin D (800 U.I. daily). Group 1 (n=15) received the supplement alone; group 2 (n=15) received the supplement in combination with HRT; group 3 (n=15) was treated with oral risedronate (35 mg weekly) and group 4 (n=15) with zoledronic acid (4 mg i.v. every 28 days for 3 months). ALP: bone alkaline phosphatase.*: $P< 0.05$ *vs*. baseline.

A significant decrease in lumbar and femoral BMD was observed in group 1 (-4.3 ± 2.3% and –4.2 ± 1.6%, P=0.046) (**Figure 2**), while a slight not significant decrease was found in group 2 (-3.1 ± 1.4% and -3.3 ± 1.5%). Risedronate treatment significantly increased lumbar BMD (5.8 ± 2.1%; P<0.035) and prevented bone loss at femoral neck (1.3 ± 1.2%; P=0.6 *vs*. baseline). Zoledronic acid increased significantly both lumbar and femoral BMD (8.6 ± 7%; P<0.01 and 5.4 ± 2.2%; P<0.039, respectively *vs*. baseline).

After the 12 month treatment period, the changes in BMD between group 4 and groups 1-2 were significant at both LS and FN (P<0.00001). Every changes between group 3 and groups 1-2 were significant at both LS and FN (P<0.00001). Only the difference in FN BMD was significant between groups 3 and 4 (P <0.0001).

The inter-group difference in absolute BMD values at 12 months was not significant.

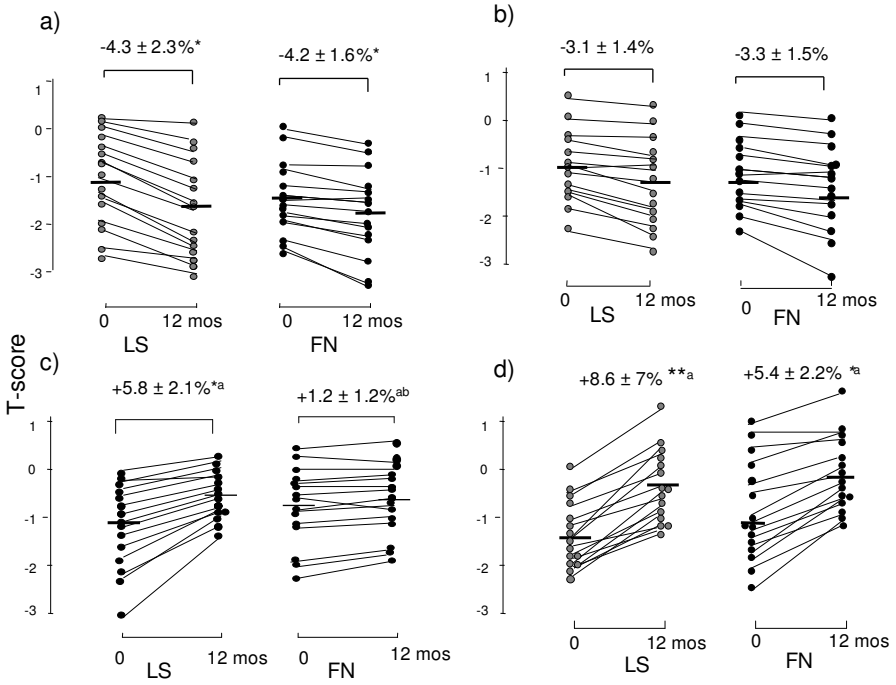


Figure 2. Densitometric values at lumbar spine and femoral neck before and 12 months after beginning of treatment, expressed as Z-scores. Groups 1-4 as in Figure 1. Horizontal bars represent mean values. *: $P< 0.05$ and ** $P< 0.01$ *vs*. baseline; [a]: $P<0.05$ *vs*. groups 1 and 2; [b]: $P<0.0001$ *vs*. group 4.

Treatment adverse effects

No patient dropped out during the 12 month treatment in any group. After HRT introduction in group 2, five women suffered from a moderate headache and six from breast tenderness. These disorders disappeared invariably within the third month of treatment. Three patients in group 3 complained about mild-to-moderate gastric pain, which was well controlled by addition of antacid treatment. Flu-like symptoms, including myalgia, body temperature increase (<37.8°C) and nausea, occurred in 12 women (80%) treated by zoledronic acid; these symptoms were well controlled using 15 mg prednisone as a pre-medication, one hour before zoledronic acid infusion.

Kidney and liver function was carefully monitored in all women every two months and did not change over the whole observation period.

Discussion

This is a controlled study comparing different anti-reabsorptive treatments in a population of hypogonadal women after allogeneic stem cell transplantation. This study clearly demonstrates that the interventions commonly used for prevention of bone loss in the general population have

no protective effects in hypogonadal transplanted women (1,22). In our experience, the supplementation of calcium and vitamin D, alone or together with the correction of hypogonadism, did not reverse bone loss in women after allo-SCT. The reasons for this may be related to the chronic and multifactorial nature of post-SCT bone loss. Indeed, age, time since SCT, degree of hypogonadism, presence of cGHVD and subsequent steroid treatments are all related in some way to the bone loss (1-5,13). Moreover, cytotoxic drugs have harmful effects on bone (23) and the transplant procedure itself negatively influences bone mass, mainly throughout "cytokine storm", GVHD and persistent reduction of the regenerative capacity of the osteoblastic bone marrow lineage (3,24,25). Considering altogether our results and the existing data on the ineffectiveness of common intervention in preventing bone loss (22), transplanted patients need to be treated by highly potent, specific agents.

Although calcium and vitamin D supplementation is insufficient by itself, it is necessary after SCT because gastrointestinal absorption of these agents is reduced and metabolism is altered (2,4). All our patients were replaced with calcium and vitamin D during the whole observational period.

Although virtually all women experience transient or permanent amenorrhea consequent to allo-SCT, little data is available on the systemic effects, the type and administration schedule of HRT. In women after the physiological menopause, HRT has been widely shown to be effective in relieving the multiple symptoms of gonadal failure, including osteoporosis (14,15). HRT was reported as effective in the treatment of post-transplant bone loss in a study by Castelo-Branco (11). We cannot confirm these results, since we could not find any improvement of BMD during the 12 month HRT administration; in fact, bone loss continued. Vasomotor symptoms and urogenital dryness disappeared in all women, whereas endocrine parameters only improved. We chose the most physiological and easy to use schedule: one pill every day for 28 days in an oral combined sequential schedule. There are three feasible explanations for the lack of HRT's protective effects in our patients: young women with precocious ovarian failure may require HRT with doses higher than those used for the treatment of physiological menopause; bone marrow transplanted women may have reduced intestinal absorption of the drugs due to persistent gastrointestinal system damage by the previous chemotherapy or to gastrointestinal cGVHD which could be mild and unrecognized. Another explanation can be the nature of post-transplant bone loss that could only partly be related to estrogen deficit.

In any case, the management of HRT in allo-transplanted women is not easy, since about 50% of patients develop chronic GVHD. HRT is contraindicated in women with abnormal liver function due to cGVHD. Little data is still available on HRT use in women who had undergone

SCT, it can be considered safe in absence of active acute or cGVHD. There is evidence that HRT does not increase the risk of cGVHD exacerbation and prevents some adverse effects of long-lasting ovarian failure in young women (26,27).

Oral bisphosphonates proved to be effective for treating post-menopausal and glucocorticoid-induced osteoporosis (14,15,17). In a previous study from our group, a 12-month treatment with a daily dose of 5 mg of risedronate increased BMD by 5.9% at the LS and prevented bone loss at the femoral neck in a mixed population of allo-transplanted patients (12). Nevertheless, gastrointestinal GVHD can impaired absorption of oral bisphosphonates, which are characterized by a low absorption from the gastrointestinal tract even in the general population (28,29). Furthermore, these agents are contraindicated in immobilized patients. Intravenous bisphosphonates proved to be effective in the treatment of postmenopausal and glucocorticoid-induced osteoporosis (30,31), although their principal indications are hypercalcemia management in cancer patients and prevention and treatment of bone metastases of various malignancies. In our experience, zoledronic acid was the only agent which improved bone density at cortical rich bone in hypogonadal females. This drug also produced beneficial effects on lumbar and femoral BMD in a mixed population of hypogonadal/eugonadal allo-transplanted men and women (13). In our center, zoledronic acid was given as three consecutive doses of 4 mg each in order to obtain a rapid and consistent effect on bone mass. Several infusion protocols have been used for the treatment of osteoporosis, but no standard regimen is currently recommended. In different patients' populations, the total dose of zoledronic acid administered over the period of one year ranged from 4 mg in menopausal women (30) to 16 mg in men with non-metastatic prostate cancer during androgen deprivation (16). These regimens caused a BMD increase that ranged from 4.3 to 5.6% at LS and from 2.7 to 3.1% at FN. A significant persistent suppression of bone turnover markers was also documented in post-menopausal women (30). An important aspect of zoledronic acid is the possibility of giving it as intermittent short-lasting infusion, which can be easily added to the complex therapeutic regimens generally used in transplanted patients.

Bisphosphonates are currently the most effective inhibitors of bone resorption; they exert a direct effect on osteoclasts, inhibiting their bone-resorbing activity (32), inducing apoptosis (32-34) and blocking their precursor proliferation (34,35). In addition, recent *in vitro* evidences have been accumulated on the anabolic bone effects of bisphosphonates. They directly enhance osteoblast proliferation and promote a differentiation of osteoprogenitor cells towards the osteoblastic pathway (36,37). Bisphosphonates also prevent osteoblast apoptosis (38) and up-regulate osteoblast production of extracellular matrix proteins (35). These effects have been proven for different members of the bisphosphonate family, including zoledronic acid,

pamidronate, neridronate, alendronate and risedronate (34,40,41). In a previous study, we documented that bone effects of zoledronic acid in transplanted patients were associated with a significant improvement in the regenerating capacity of osteogenic stromal lineage precursors. Similar findings have also been reported for risedronate *in vitro:* risedronate stimulated osteoblast proliferation in a human trabecular bone culture model. This effect was more evident and sustained over a wide range of time periods and drug concentration when produced by risedronate as opposed to alendronate (40).

The positive effects on osteoblasts may be particularly important in allo-transplanted patients, since previous evidence indicated a persistently reduced regenerative and functional capacity of osteoblast precursors (3,23,24,41,42). Although we did not evaluate osteoblastic lineage behavior in patients treated by risedronate, it is likely that BMD improvement produced by risedronate is at least in part related to its anabolic effects, in analogy with what we documented with zoledronic acid (13). As a matter of fact, the improvement observed in the patients treated by bisphosphonates was highly significant at both LS and FN. A significant decrease in hydroxyproline excretion was also observed in the groups treated by bisphosphonates. An increase in serum bone alkaline phosphatase and osteocalcin was just at the limit of statistical significance only in the zoledronate group. When concerning with bone formation markers in post-transplant osteoporosis, controversial results were reported in serum osteocalcin levels during bisphosphonate treatment (43,44).

Side effects of the treatments employed were generally mild and well controlled by appropriate pre-medications. No alteration in kidney function was detected at any time point during the study, and liver enzymes did not worsen during the treatment period.
No conclusion can be made about fracture risk reduction because this study is not sufficiently powerful to evaluate fracture incidence as a clinical end-point. Whether any treatment is able to reduce fracture risk after allo-SCT still remains to be determined.

In conclusion, persistent multifactorial bone loss in allo-transplanted patients needs a specific and careful treatment. Neither calcium plus vitamin D supplementation nor its association with HRT were able to prevent bone loss in young adult hypogonadal women after allo-SCT. Bisphosphonates are currently the only treatment able to prevent and treat bone demineralization in this particular population of patients. Weekly oral risedronate and monthly i.v. zoledronic acid infusion were easily manageable and effective in increasing densitometric values at trabecular skeletal site. Zoledronic acid was the only therapy that improved femoral BMD. At least part of these effects can be ascribed to the bisphosphonates' ability in reversing the persistent post-transplant reduction of clonogenic fibroblast progenitors belonging to the

osteogenic stromal lineage. Currently, the choice between oral or intravenous bisphosphonate therapy in patients who had undergone allogeneic SCT should be made on the basis of individual clinical conditions, including presence, grading and localization of GVHD, and prevalent site of bone loss. The evaluation of cost-effectiveness requires the data on fracture prevention that still have to be produced.

References

1. Valimaki MJ, Kinnunen K, Volin L, Tahtela R, Loyttyniemi E, Laitinen K, Makela P, Keto P, Ruutu T. A prospective study of bone loss and turnover after allogeneic bone marrow transplantation: effect of calcium supplementation with or without calcitonin. *Bone Marrow Transplant* 1999; **23:**355-361.
2. Stern JM, Sullivan KM, Ott SM, Seidel K, Fink JC, Longton G, Sherrard DJ. Bone density loss after allogeneic hematopoietic stem cell transplantation: a prospective study. *Biol Blood Marrow Transplant* 2001; **7**: 257-264.
3. Tauchmanovà L, Serio B, Del Puente A, Risitano AM, Esposito A, De Rosa G, Lombardi G, Colao A, Rotoli B, Selleri C. Long-lasting bone damage detected by dual energy x-ray absorptiometry, phalangeal osteosonogrammetry and *in vitro* growth of marrow stromal cells after allogeneic stem cell transplantation. *J Clin Endocrinol Metab* 2002; **87**: 5058-5065.
4. Gandhi MK, Lekamwasam S, Inman I, Kaptoge S, Sizer L, Love S, Bearcroft PW, Milligan TP, Price CP, Marcus RE, Compston JE. Significant and persistent loss of bone mineral density in the femoral neck after haematopoietic stem cell transplantation: long-term follow-up of a prospective study. *Br J Haematol* 2003; **121**: 462-468.
5. Schimmer AD, Minden MD, Keating A. Osteoporosis after blood and marrow transplantation: clinical aspects. *Biol Blood Marrow Transplant* 2000; **6**:175-181.
6. Weilbaecher KN. Mechanisms of osteoporosis after hematopoietic cell transplantation. *Biol Blood Marrow Transplant* 2000; **6**:165-171.
7. Schulte CMS, Beelen DW. Bone loss following hematopoietic stem cell transplantation: a long-term follow-up. *Blood* 2004; **103**:3635-3643.
8. Brennan BMD & Shalet SM. Endocrine late effects after bone marrow transplant. [Review]. *Br J Haematol* 2002; 118:58-66.
9. Chatterjee R, Kottaridis PD. Treatment of gonadal damage in recipients of allogeneic or autologous transplantation for haematological malignancies. Mini-review. *Bone Marrow Transplant* 2002; 30:629-635.
10. Tauchmanovà L, Selleri C, De Rosa G, Esposito M, Di Somma C, Orio F Jr, Lombardi G, Rotoli B, Colao A. Gonadal status in reproductive age women after allogeneic or autologous stem cell transplantation. *Hum Reprod* 2003, 18:1410-1416.
11. Castelo-Branco C, Rovira M, Pons F, Duran M, Sierra J, Vives A, Balasch J, Fortuny A, Vanrell J. The effect of hormone replacement therapy on bone mass in patients with ovarian failure due to bone marrow transplantation. *Maturitas* 1996; **23**:307-312.
12. Tauchmanovà L, Selleri C, Esposito M, Di Somma C, Orio F Jr, Bifulco G, Palomba S, Lombardi G, Rotoli B, Colao A. Beneficial treatment with risedronate in long-term survivors after allogeneic stem cell transplantation for hematological malignancies. *Osteoporosis International*, 2003, 14:1013-1019.
13. Tauchmanovà L, Ricci P, Serio B, Colao A, Lombardi G, Rotoli B & Selleri C. Effect of intravenous zoledronic acid administration in patients with low bone mineral density and/or fast bone loss after allogeneic stem cell transplantation. *J Clin Endocrinol Metab*, 2005, 90: 627-634.
14. Marcus R, Wong M, Heath H III, Stock JL. Antiresorptive treatment of postmenopausal osteoporosis: comparison of study designs and outcomes in large clinical trials with fracture as an endpoint. *Endocr Review* 2002; **23**:16-37.
15. Delmas PD. Treatment of postmenopausal osteoporosis. *Lancet* 2002; **8:**2018-2026.

16. Smith MR, Eastham J, Gleason DM, Shasha D, Tchekmedyian S, Zinner N. Randomized controlled trial of zoledronic acid to prevent bone loss in men receiving androgen deprivation therapy for nonmetastatic prostate cancer. *J Urol* 2003; 169:2008-2012.

17. Cohen D, Adachi JD. The treatment of glucocorticoid-induced osteoporosis. *J Steroid Biochem Mol Biol* 2004; 88:337-349.

18. Lee SJ, Vogelsang G, Flowers ME. Chronic graft-versus-host disease. Biol Blood Marrow Transplant. 2003;9:215.

19. del Puente A, Heyse SP, Mandes MG, Mantova D, Carpinelli A, Nutile G, Oriente P. Epidemiology of osteoporosis in women in southern Italy. *Aging Clin Exp Res* 1998; **10**: 53-58.

20. Minne HW, Leidig G, Wüster C, Siromachkostov L, Baldauf G, Bickel R, et al. A newly defined spine deformity index (SDI) to quantitative vertebral crush fractures in patients with osteoporosis. *Bone Miner J* 1988, **3**: 335-349.

21. World Health Organization Study Group. (1994) Assessment of fracture risk and its application to screening for postmenopausal osteoporosis. WHO Technical Report Series 843. WHO, Geneve

22. Massenkeil G, Fiene C, Rosan O, Michael R, Reisinger W, Arnold R. Loss of bone mass and vitamin D deficiency after hematopoetic stem cell transplantation: standard prophylactic measures fail to prevent osteoporosis. *Leukemia* 2001; **15**:1701-1705.

23. Banfi A, Podesta M, Fazzuoli L, Sertoli MR, Venturini M, Santini G, Cancedda R, Quarto R. High-dose chemotherapy shows a dose-dependent toxicity to bone marrow osteoprogenitors: a mechanism for post-bone marrow transplantation osteopenia. *Cancer* 2001; **92**:2419-2428.

24. Lee WY, Cho SW, Oh ES, Oh KW, Lee JM, Yoon KH, Kang MI, Cha BY, Lee KW, Son HY, Kang SK, Kim CC 2002 The effect of bone marrow transplantation on the osteoblastic differentiation of human bone marrow stromal cells. *J Clin Endocrinol Metab* 2002; **87:** 329-335.

25. Stern JM, Chesnut CH III, Bruemmer B, Sullivan KM, Lenssen PS, Aker SN, Sanders J. Bone density during treatment of chronic GVHD. *Bone Marrow Transplant* 1996; **17:**395-400.

26. Balleari E, Garre S, Van Lint MT, Spinelli S, Chiodi S, Repetto E, Massa G, Bacigalupo A, Ghio R. Hormone replacement therapy and chronic graft-versus-host disease activity in women treated with bone marrow transplantation for hematologic malignancies. *Ann N Y Acad Sci* 2002;966:187-192.

27. Chatterjee R, Kottaridis PD. Treatment of gonadal damage in recipients of allogeneic or autologous transplantation for hematological malignancies. *Bone Marrow Transplant* 2002; 30:629-635.

28. Okolicsanyi L, Dal Bo N. The gastrointestinal tolerability of bisphosphonates. *Aging Clin Exp Res* 2001;13:344-346.

29. Adami S, Zamberlan N. Adverse effects of bisphosphonates - a comparative review. *Drug Saf* 1996;14:158-170.

30. Reid IR, Brown JP, Burckhardt P, Horowitz Z, Richardson P, Trechsel U, Widmer A. Intravenous zoledronic acid in postmenopausal women with low bone mineral density. *N Engl J Med* 2002; **346**:653-661.

31. Boutsen Y, Jamart J, Essenlinckx W, Devogelaer JP. Primary prevention of glucocorticoid-induced osteoporosis with intravenous pamidronate and calcium: a prospective controlled 1-year study comparing a single infusion, an infusion given once every 3 months, and calcium alone. *J Bone Miner Re*s 2001;16:104-112.

32. Selander K, Lehenkari P, Väänänen HK. The effects of bisphosphonates on the resorption cycle of isolated osteoclasts. *Calcif Tissue Int* 1994; **55***:*368-375, 1994.

33. Rogers M J, Chilton KM, Coxon FP, Lawry J, Smith MO, Suri S, Russell RG. Bisphosphonates induce apoptosis in mouse macrophage-like cells in vitro by a nitric oxide-independent mechanism. *J Bone Miner Res* 1996; **11:** 1482-1491.

34. Reszka AA, Halasy-Nagy JM, Masarachia P J, Rodan GA. Bisphosphonates act directly on the osteoclast to induce caspase cleavage of Mst1 kinase during apoptosis. *J Biol Chem* 1999; **274**: 34967-34973.

35. Reinholz GG, Getz B, Pederson L, Sanders ES, Subramaniam M, Ingle JN, Spelsberg TC. Bisphosphonates directly regulate cell proliferation, differentiation, and gene expression in human osteoblasts. *Cancer Res.* 2000; **60**:6001-6007.

36. Fromigue O & Body JJ. Bisphosphonates influence the proliferation and the maturation of normal human osteoblasts. *J Endocrinol Invest* 2002; **25**:539-546.

37. Plotkin LI, Weinstein RS, Parfitt AM, Roberson PK, Manolagas SC, Bellido T. Prevention of osteocyte and osteoblast apoptosis by bisphosphonates and calcitonin. *J Clin Invest* 1999; **104:** 1363-1374.

38. Giuliani N, Pedrazzoni M, Negri G, Passeri G, Impicciatore M, Girasole G. Bisphosphonates stimulate formation of osteoblast precursors and mineralized nodules in murine and human bone marrow cultures in vitro and promote early osteoblastogenesis in young and aged mice in vivo. *Bone* 1998; **22:** 455-461.

39. Im GI, Qureshi SA, Kenney J, Rubash HE, Shanbhag AS. Osteoblast proliferation and maturation by bisphosphonates. *Biomaterials* 2004; **25**: 4105-4115.

40. Frediani B, Spreafico A, Capperucci C, et al. Long-term effects of neridronate on human osteoblastic cell cultures. *Bone* 2004; 35;859-869.

41. Galotto M, Berisso G, Delfino L, Podestà M, Ottagio L, Dallorso S, Dufour C, Ferrara GB, Abbondandolo A, Dini G, Bacigalupo A, Cancedda R, Quarto R. Stromal damage as consequence of high dose chemo/radiotherapy in bone marrow transplant recipients. *Exp Hematol* 1999; **27**, 1460-1466.

42. Tauchmanovà L, De Rosa G, Serio B, Fazioli F, Mainolfi C, Lombardi G, Colao A, Salvatore M, Rotoli M, Selleri C. Avascular necrosis in long-term survivors after allogeneic or autologous stem cell transplantation: a single center experience and a review. *Cancer* 2003;97:2453-2461.

43. Cruz DN, Brickel HM, Wysolmerski JJ, Gundberg CG, Simpson CA, Kliger AS, Lorber MI, Basadonna GP, Friedman AL, Insogna KL, Bia MJ. Treatment of osteoporosis and osteopenia in long-term renal transplant patients with alendronate. *Am J Transplant* 2002; **2**: 62-67.

44. Haas M, Leko-Mohr Z, Roschger P, Kletzmayr J, Schwarz C, Mitterbauer C, Steininger R, Grampp S, Klaushofer K, Delling G, Oberbauer R**.** Zoledronic acid to prevent bone loss in the first 6 months after renal transplantation. *Kidney Int* 2003; 63:1130-1136.

Discussion

Haematopoietic stem cell transplant has become a successful treatment for different hematological malignancies and other diseases as well, and is generally performed in young patients. Multiple late complications have already been described but little information on risk factors of these long term effects of SCT is available, except for gonadal failure. Moreover, frequent post-transplant endocrine and bone disorders raise an important clinical question of when and how to treat them. Appropriate diagnosis and treatment of post-transplant complications can consistently improve the quality of life and general health in long-term sirvovors after SCT.

Endocrine disorders were mostly ascribed to highly aggressive chemotherapy and TBI. However, multiple dysfunctions were found in the endocrine system of patients not treated by TBI, even years after SCT. While the toxic effects of antiblastic agents have been widely described, the impact of immune system damage on endocrine function is less clear. We have focused our attention on the hypothesis that the type of transplant can be important for endocrine and bone disorders. In particular, thyroid and adrenal functions, and IGF-1 impairment occur more frequently in patients after allografting than in those who have undergone auto-SCT. We have demonstrated that cGVHD profoundly impairs endocrine functions in allo-SCT recipients, also in the absence of TBI. Allogeneic bone marrow/blood derived stem cell transplantation is the only condition in which a great amount of donor immunocompetent cells are infused to a host that is recognized as “nonself”, and thus causing graft-versus–tumor reaction, and GVHD. While the first is necessary for disease disappearance, the second represents a frequent complication that requires immunosuppressive treatments, which can further deregulate the function of the immune system. Patients developing cGVHD seem to be at a greater risk for endocrine dysfunction, including an increased frequency of thyroid disorders, adrenal insufficiency and IGF-I reduction. Their residual gonadal function also seem to be worse. These findings suggest that immunosuppressive treatments and immune system derangement play an important role in the development of endocrine dysfunction after allografting.

Precocious gonadal failure, adrenal insufficiency, thyroiditis and hypothyroidism require specific monitoring and treatment by specialized operators. Therefore, patients affected by these disorders require life-long monitoring to detect, prevent and treat their symptoms.

Moreover, we found a greater increase in serum leptin after allo-SCT than after auto-SCT, with the highest levels in patients affected by cGVHD. The physiological relationship between leptin, body mass and gonadal function was lost in allotransplanted setting, while a close relationship between leptin and IFN-gamma was documented in allotransplanted patients. A closer relationship between these two parameters was observed in subjects with increased IFN-gamma, suggesting that a higher degree of immune system activation can trigger leptin production in the post-transplant period. Since leptin regulates the balance of Th1/Th2 cytokines, it is possible that Th1 lymphocyte activation during cGVHD may account for the difference in leptin values observed between auto- and allo-transplanted subjects. A major increase in serum leptin in patients with cGVHD suggests that autoreactive T cells involved in cGVH reaction may further stimulate leptin secretion. A minor increase in serum leptin levels and preserved leptin/BMI ratio observed after auto-SCT are in line with a milder immune system alteration in this cohort of patients. These results further confirm a close relationship between the immune and endocrine systems.

When regards to bone loss conseguent to SCT, patients who underwent allogeneic transplant are at a higher risk for bone loss than those autotransplanted. Bone mass at either cancelous or cortical rich sites usually improves with time after autografting, while allotransplanted patients can continue to lose cortical bone even years after the transplant. Our treatment experience in the allogeneic setting clearly confirms prior evidence on the inability of conventional interventions used to prevent bone loss in the general population in transplanted patients. The supplementation of calcium and vitamin D, alone or together with the correction of hypogonadism, did not reverse bone loss in our women after allo-SCT. The reasons for this may be related to the chronic and multifactorial nature of post-SCT bone loss. Indeed, age, time elapsed since SCT, degree of hypogonadism, presence of cGHVD and subsequent steroid treatments are all in some way related to bone loss. Moreover, cytotoxic drugs have harmful effects on the bone, and the transplant procedure itself negatively influences bone mass, mainly throughout “cytokine storm,” GVHD and persistent reduction of the regenerative capacity of the osteoblastic bone marrow lineage. Considering our results altogether with the existing data on the ineffectiveness of common intervention in preventing bone loss, transplanted patients need to be treated by highly potent and specific agents. The most potent agents that are currently available on the market for the treatment of osteoporosis are bisphosphonates. Although they are considered as antireabsorbtive drugs, a huge amount of evidence has been accumulated *in vitro,* showing that they stimulate proliferation and differentiation of the osteoblastic precursors at lower concentrations. On the contrary, at

higher concentrations, the bisphoshonates have prevalent inhibitory effects on osteoclasts. For the first time, we have documented a significant improvement of the repopulation capacity of osteoblastic precursors *in vitro* that was obtained by the administration of zoledronic acid *in vivo*. The anabolic effect on osteoblasts can be particularly useful in allotrasnplanted patients that have inhibited the repopulating capacity of the osteoblastic lineage.

Conclusion

Complications of the endocrine and muscoloschelettal systems were more frequent, more severe and more closely related to the degree of immunological deregulation in the allogeneic setting than in the autologous one. If they are left untreated, they can worsen the quality of life and sometimes shorten the overall survival.

Persistent endocrine disorders were observed in the allogeneic setting even years after the transplant. Frequent endocrine disorders were revealed during the first year after autologous stem cell transplant; despite the tendency to improve, in more than half of the cases, the complications persisted over one year. Multiple risk factors including previous antiblastic, corticosteroid and radiation treatments, an abnormal immunological condition and general health status are likely responsible for the onset of endocrine disorders.

Persistent bone loss at cortical bone has a multifactorial origin in patients after allogeneic stem cell transplant, and seems to be in great part related to the transplant procedure itself. It tended to improve in patients cured from the underlying disease by the autolgous SCT. Bone density should be monitored in all subjects during the first year after autologous SCT. Bone loss should be periodically investigated in all allotransplanted patients during life-long follow-up. Any significant decrease in bone mass should be treated by specific potent agents with a possible anabolic effect on the osteoblastic production.

In conclusion, periodical screening for endocrine disorders and bone loss is necessary in long-term survivors after allogeneic stem cell transplant. In the autologous setting, all the patients should be evaluated for both endocrine disorders and bone loss at least during the first year after transplant and thereafter, according to their clinical conditions, underlying disease and previous treatments.