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Review

Non-clonal chromosome aberrations and genome chaos in somatic and germ cells from patients and survivors of Hodgkin lymphoma, induced by anticancer treatment

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Abstract: Anticancer regimens for Hodgkin lymphoma (HL) patients include highly genotoxic drugs that have been very successful in killing tumor cells and providing a 90% disease-free survival at five years. However, these treatments do not have a specific cell target, damaging both cancerous and normal cells. Thus, HL survivors have a high risk of developing new primary cancers, both hematologic and solid tumors, that have been related to treatment. Several studies have shown that after-treatment, HL patients and survivors present persistent chromosomal instability, including non-clonal chromosomal aberrations. The frequency and type of chromosomal abnormalities appear to depend on the type of therapy and the cell type examined. For example, MOPP chemotherapy affects hematopoietic and germ stem cells leading to long-term genotoxic effects and azoospermia, while ABVD chemotherapy affects transiently sperm cells, with most of the patients showing recovery of spermatogenesis. Both regimens have long-term effects in somatic cells, presenting non-clonal chromosomal aberrations and genomic chaos in a fraction of non-cancerous cells. This is a source of karyotypic heterogeneity that could eventually generate a more stable population acquiring clonal chromosomal aberrations and leading towards the development of a new cancer.

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Keywords: Chromosome instability (CIN); Chromoplexy; Genome chaos; Chromosomal heterogeneity; Karyotype heterogeneity; Non-clonal chromosome aberration (NCCA); Second cancer

1. Introduction

The population of cancer survivors in the world is continuously growing. In 2016, in the United States there was a population of survivors of more than 15 million people and it is projected that by 2026 there will be more than 20 million in this country alone [1]. This is due to several factors, including that the population is growing and aging, that there is better and earlier detection of cancer, and, importantly, to the great success and effectiveness of anticancer therapies.

One of the cancers with a high likelihood of cure is Hodgkin's lymphoma (HL), which is a neoplasm affecting the B-cells of the lymphoid system and has an average annual age-adjusted incidence rates of 3.2, 2.5, 2.3 and 1.3 per 100,000 in Whites, Blacks, Hispanics and Asians, respectively, however, the major variation in the incidence is by the age at diagnosis, with more than a half of the cases occurring during the teenage years or early 20s [2,3]. Among adolescents, HL is the third most frequent cancer, preceded only by brain/CNS cancer and leukemia [1].

The survival rate for HL patients is near 90% at five years [1]. However, this high survival rate is associated with secondary events to treatment. In fact, survivors of all types of childhood cancers have a higher risk for subsequent hospitalization and spend five times as many days as compared with healthy individuals of similar age. In particular, HL survivors are among those with the highest risk of presenting with a new cancer and the highest number of days of hospitalization. In addition, approximately half of these days spent in the hospital are due to recurrences, but also to new primary cancers [4]. These long-term complications are likely due to the use of strong genotoxic agents as part of the anticancer treatment.

The purpose of this review is to provide a brief overview of the available data related on the genotoxic consequences of the aggressive and genotoxic anticancer treatment used in HL patients. The focus is on therapy-induced chromosomal abnormalities that have been considered as partially responsible for the azoospermia and oligospermia in male patients, as well as the development of new neoplasms that are observed in 10-20% of the HL survivors.

2. Genotoxicity of the anticancer treatment in Hodgkin lymphoma

In general, the treatment strategy for HL consists of a combination of chemotherapy and radiotherapy (RT). There are several chemotherapy regimens that include a mixture of agents that are efficient in killing cancer cells (Table 1), in recent decades, the most used regimens are: MOPP (Mechlorethamine, Oncovin, Procarbazine, Prednisone); NOVP (Novantrone, Oncovin, Vinblastine, and Prednisone); COPP (Cyclophosphamide, Oncovin, Procarbazine, Prednisone); and, ABVD (Adriamycin, Bleomycin, Vinblastine and Dacarbazine). These treatments include cytotoxic and genotoxic chemicals that affect tumor cells by damaging the DNA and interfering with the processes of DNA replication and/or repair, or altering the processes of chromosome segregation during cell division (Table 1) [5,6].

In the 1960s, the first effective chemotherapy for HL was the MOPP regimen that included alkylating agents such as nitrogen mustard and procarbazine, which are recognized as potent clastogenic and mutagenic agents [7,8]. This regimen was effective in the treatment of advanced HL with or without radiation therapy, with a 65-70% survival five years after treatment, however, it had high reproductive toxicity and great carcinogenic potential. Since the 70s, several modifications

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to the MOPP regimen were introduced to maintain chemotherapeutic efficacy and reduce associated toxic effects. New schemes such as ABVD, NOVP or mixtures of MOPP/ABVD were developed to avoid high doses of alkylating agents, produce fewer side effects, lower the incidence of secondary cancers and achieve an excellent recovery of reproductive function. Currently, the ABVD scheme is the therapeutic standard in HL, containing fewer alkylating agents and still providing adequate elimination of tumor cells while resulting in an excellent disease-free survival greater than 85% [9].

Treatment of HL includes the use of RT at the total dose used 40-44 Gy when it is applied after chemotherapy [10,11], the total dose used is 40-44 Gy, while in combination with chemotherapy the total dose is reduced to 20-30 Gy [12,13]. Ionizing radiation is very genotoxic, directly breaks the DNA molecule generating single and double strand breaks, base damage, crosslinks, dicentric chromosomes, acentric fragments, inversions, and stable chromosomal rearrangements such as translocations. Another type of damage induced by RT in HL patients is the inhibition of mitotic activity of lymphocytes as well as a high frequency of micronuclei and sister chromatids exchanges [14].

Table 1. Chemical compounds used in chemotherapy with genotoxic effect on somatic and germinal cells.

Type	Drug	DNA lesion	Altered mechanism	Cytogenetic alterations
Alkylating agents (Monofunctional and Bifunctional)	**Nitrogen mustard (Mechlorethamine)** **Dacarbazine** **Procarbazine** Mitomycin C Cyclophosphamide Ethyl-nitrosourea Melphalan Cisplatin Ifosphamide Clorambucil	Base damage Bulky adducts DNA intrastrand crosslinks DNA interstrand crosslink Double strand breaks	Interferes with DNA synthesis	Chromosomal deletions, insertions, inversions and translocations
Antibiotics	**Doxorubicin (Adriamycin)** Daunorubicin Epirubicin Idarubicin **Bleomycin**	Free radicals Crosslink DNA Single strand breaks Double strand breaks Intercalant of DNA	Blocking of DNA replication and transcription	Chromatid and chromosome-type aberrations, translocations, dicentric, acentric, and other aberrations related to damage of telomere
Mitosis inhibitors	**Vincristine (Oncovin)** **Vinblastine** Vinorelbine	Induce aneuploidy	Interference with tubulin polymerization	Aneuploidy and polyploidy

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			and inhibits mitotic spindle	
Topoisomerase II inhibitors	Daunorubicin Epirubicin **Mitoxantrone** (**Novantrone**) Camptothecin Etoposide	Single strand breaks Double strand breaks Replication lesions	Inhibition of DNA synthesis by forming a complex with Topo II and DNA	Chromosomal translocations, aneuploidy, polyploidy and endoreduplication

In bold, the drugs used in chemotherapy for HL

3. Risk for a new cancer in Hodgkin lymphoma patients.

Anticancer treatments do not have a specific cell target, damaging the genetic material of both normal and cancerous cells. Induced genetic damage may be lethal and non-lethal. When occurring in somatic cells such as blood cells, lethal damage can generate anemias or infections, while in germ cells it can produce oligospermia and transitory or permanent azoospermia. Importantly, non-lethal damage can also have serious consequences, with surviving cells carrying numerical and structural chromosomal damage that in somatic cells could causes secondary cancers related to treatment, and in germ cells could result in abortions or offspring with genetic affectations [15,16].

The therapeutic regimens used in HL patients, with multi-agent chemotherapy (CT) and RT, have resulted in a population of young people who due to the stress of genotoxic anticancer treatment, have a high risk of developing a new and different cancer [17,18]. The estimated risk of secondary cancer is 43.6% with a 40-year cumulative incidence [19]. There are three main types of new primary cancer in HL survivors: non-Hodgkin lymphoma is reported in 17% of patients, 25% leukemia in CT treated patients and 58% of solid tumors in CT+RT treated HL patients. Breast, lung and colorectal cancer are the most common solid malignancies after chemotherapy treatment of HL [18]. A strong correlation has been observed between the dose of RT and the radiation field size with the development of secondary solid tumors [19]. Associated with CT, alkylating agents have been related with the development of therapy-related myelodysplastic syndrome and acute myeloid leukemia [17,20].

4. Genomic instability, chromosome instability and genomic chaos.

Genomic instability is a condition in which the genomes of a specific tissue or organism are constantly generating genetic alterations, both at a small scale, such as changes of a single nucleotide, deletions, duplications, indels, microsatellite instability, or, at large scale, such as gain or loss of whole chromosomes, translocation, inversions etc. When genomic instability occurs at the chromosome level, then it is called chromosomal instability (CIN), which is characterized by an increased rate of karyotype variability in a given cell population, and thus, cell to cell variability [21].

CIN may be intrinsic (constitutional), associated with germinal mutations in genes related to chromosomal segregation. In these cases, all cells of the individual are prone to genomic instability. For example, mutations in genes such as BUBR1 and BUB1B cause mosaic variegated aneuploidy [22], while mutations in genes related to DNA repair or response to DNA damage, such as FANC genes in Fanconi anemia, or ATM in Ataxia Telangiectasia [23,24] cause syndromes displaying CIN

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at the cellular level and an increased risk of cancer [21,25]. However, these types of mutations are rare and do not explain most sporadic cancers.

CIN may be also extrinsic, related to non-genetic factors such as chemotherapy or radiation exposure, virus infection and some physiologic processes like inflammation or aging; these extrinsic mechanisms of CIN are frequently associated with sporadic cancer [21,25]. Both CIN originated by intrinsic or extrinsic mechanisms, have the common feature of generating heritable chromosomal variation. The stress-produced heterogeneous population of cells make the genomic system unstable, producing new chromosomal combinations that may drive toward adaptation and then evolution of cancer [21,25,26].

CIN can manifest as numerical CIN, consisting of gains or losses of whole chromosomes and structural CIN characterized by chromosomal rearrangements such as deletions, duplications, translocations, isochromosomes, dicentrics, complex rearrangements, massive rearrangement of the genome or genome chaos, and others [21,27]. Structural CIN can occur due to template switching or, by erroneously repaired double strand breaks (DSB) in the DNA [28]. The greater the number of DSBs, the more frequent and more complex structural chromosomal alterations are originated (Table 2, Figure 1). Both numerical and structural CIN can be found in the same cell population and coexist in a single cell; whole or segmental aneuploidy are a by-product of CIN [29].

CIN can be identified as non-clonal chromosomal alterations (NCCA) present in a population of cells in a non-recurrent manner, thus creating a heterogeneous cell population with a specific chromosomal rearrangement frequency of less than 4% among 50–100 mitosis [30]. CIN may also be represented by clonal chromosomal alterations (CCA), which are recurrent chromosomal alterations that are found at least twice in a population of 20-40 mitosis, or in more than 5% of the cells. Thus, NCCA reflect a more dynamic genome system, and is a better indicator of CIN, while CCA reflects a more stable system [21,26].

Another representative event of CIN is genomic chaos, which is a massive reorganization of the genome in the cell. Karyotypic chaos is triggered by one event of cellular crisis leading to chromosome fragmentation with the excess of DSBs generating extreme structural rearrangements such as chromotripsis, defined by multiple rearrangement occurring within a chromosome, and chromoplexy, another type of genome chaos consisting in reshuffling the genetic material among several chromosomes, generating multiple translocations among multiple chromosomes (Figure 1). A cell population may be recognized as having genome chaos when it presents a highly heterogeneous cell population with NCCA, and a number of cells carrying chromotripsis and chromoplexy [31].

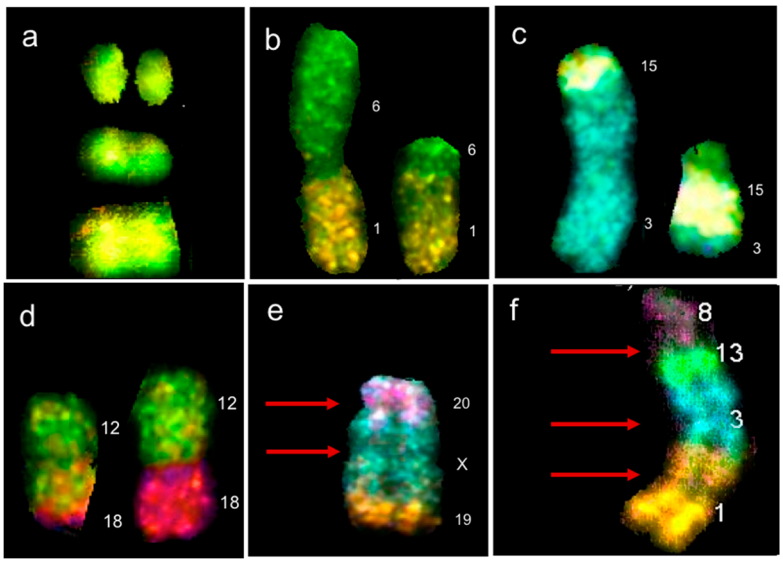

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Figure 1. Chromosomal abnormalities found by M-FISH in lymphocytes of HL survivors. a) Chromosome 12 with two DSB in different arms of the same chromosome; b) Balanced translocation t(1;6)(p?;p?); c) Balanced translocation t(3;15)(p?;q?); d) Balanced translocation t(12;18)(q?;p?); translocations in b), c) and d) resulting from erroneous DNA repair of two DSBs occurring on two non-homologous chromosomes; e) Rearrangement Dicentric + deletion + translocation, resulting from 4 DSBs, two on the same chromosome X, one on chromosome 20 and one on chromosome 19; d) Complex rearrangement resulting from multiple DSBs on multiple chromosomes, found in a cell with chaotic karyotype (chromoplexy). Red arrows represent centromeres, numbers represent the chromosomes involved in the rearrangement [32].

Table 2. Complexity and types of rearrangements according with the number of co-ocurring DSBs

Number of DSBs	Location of the DSBs	Possible Rearrangements
2	DSBs occurs in the same arm of the same chromosome	a) Deletion + ring or + acentric fragment b) Paracentric inversion
	DSBs occurs in the different arms of the same chromosome	c) Ring with deletion + acentric fragment d) Pericentric inversion
	DSBs occur in the same arm of two homologous chromosomes	e) Translocation, interstitial duplications and deletions f) Dicentric chromosome with deletion + acentric fragment
	DSBs occur on different, nonhomologous chromosomes	g) Balanced translocation h) Dicentric chromosome + acentric fragment
3	All the anterior with two DSBs (a-h)	i) All the anterior with two DSBs (a-h) + one deletion or + one inversion
	DSBs occur on three different non-homologous chromosomes	j) Complex three-way balanced translocation k) Dicentric + deletion + acentric fragment
	Two DSBs on the same chromosome and one DSB on a different chromosome	l) Inversion + deletion + acentric fragment m) Interchromosomal insertion
Multiple	DSBs occur in the same chromosome	n) Chromotripsis, genomic chaos
	DSBs occur in two different chromosomes	o) Chromotripsis, genomic chaos

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DSBs	DSBs occur in multiple different chromosomes	p) Chromoplexy, complex chromosome rearrangements, multiple deletions and duplications, genomic chaos

NCCA are an indisputable reflection of CIN and karyotypic heterogeneity. In a genomic or karyotypic system that presents genomic instability, each cell may have a different potential for survival and evolution while facing environmental challenges. Thus, by classical mechanisms of natural selection, some chromosomal alterations may become clonal (present in more than 5% of the cell population) and transient. However, some CCAs can be selected and persist if they confer an adaptive advantage, which is generally related to a greater resistance to the adverse environment and to conferring a reproductive advantage; this generates an NCCA / CCA generation cycle that can lead to the formation of a neoplasm [33].

Experimentally, it has been tested in vitro that chemotherapeutic agents such as Doxorubicin and Mitomycin-C, induce high chromosomal damage of the NCCA type, such as complex chromosomal alterations, chromotripsis, chromoplexy and other cytogenetic alterations like heterogeneous chromosomal condensation or fragmentation [31]. The experiments of Liu and colleagues have clearly shown that drugs used as anticancer agents induce primarily NCCA and karyotypic chaos, and that this karyotypic heterogeneity is essential for the cell population to survive and adapt to chemical stress, giving rise to more stable karyotypic systems with high survival and reproduction capacity in an adverse environment. This cellular population generated by the stress of anticancer drugs, presents heterogeneous karyotypes accompanied by transcriptome and phenotype heterogeneity. Importantly, these cells retain cellular heterogeneity by maintaining the NCCA through a "fuzzy inheritance", which is a strong strategy to survive because it has a large chance to produce a large number of potential survivors, most of which are distinctively different [34]. This condition has been linked to the punctuated phase of cancer evolution and, indeed, NCCAs are present in the key transition stages of cancer evolution such as immortalization, transformation, metastasis and drug resistance [33].

5. Impact of anticancer therapy in non-cancerous somatic cells from patients with Hodgkin lymphoma

There are several studies showing that chemotherapy (CT)/RT has a genotoxic effect on somatic cells from HL patients [17,20,35–40]. Smith and colleagues [35] detected the genotoxic effects induced by MOPP/RT treatment through painting of chromosome 4 in lymphocytes from patients who were treated 12 to 24 years before the study. They found an increase in chromosomal translocations up to 24 years after treatment, which suggested that anticancer treatment induced permanent damage in the bone marrow hematopoietic cells. In another study, using chromosome banding analysis, it was found a significant increase in chromosome breaks, acentric fragments, dicentrics, and rings in the lymphocytes from HL patients who received MOPP/ABV and RT, which persisted six months after treatment [14]. Similarly, in lymphocytes of HL patients treated with BEACCOP, EBVP, ABVD, MOPP/ABV, using FISH analysis with painting probes for chromosomes 1, 3 and 4, M'Kacher *et al*. [37] observed an increase in the frequency of structural chromosomal rearrangements before CT and two years later, a significant increase in complex chromosomal rearrangements involving several breaks and more than two chromosomes [37].

The persistence of structural chromosome rearrangements was confirmed by Salas *et al*. [17], who studied the genotoxic consequences of the MOPP/with or without RT CT in 20 HL patients 2-17

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years after the therapy stress. In this study, 1000 lymphocytes in metaphase per patient were analyzed with G banding and 13 out of 20 survivor patients were found to have a high frequency of breaks and NCCA chromosomal structural rearrangements (Figure 2).

Most of the aberrations were non-clonal, with a unique or multiple alterations per cell, consistent with persistent CIN as a result of anticancer treatment. Only one patient showed a CCA structural consisting in a deletion del(17)(p11.2p11.2) in three cells [17]. The majority of chromosomal rearrangements found in this group of HL survivors were NCCA, within a little population of cells with genomic variation that could be detected only because a large number of cells were studied (Figure 2).

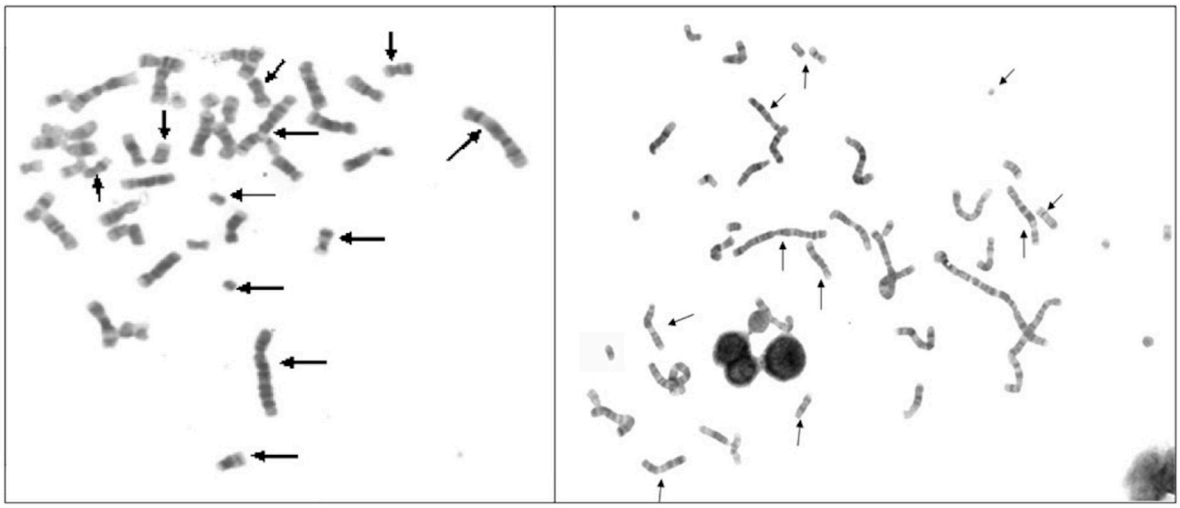

Figure 2. NCCA observed in a group of 20 HL survivors up to 17 years after anticancer treatment MOPP, with or without radiotherapy. a) Metaphase in peripheral blood lymphocytes from a HL patient 2 years after MOPP treatment, b) Metaphase from a HL survivor 13 years after MOPP treatment [17]. Arrows indicate abnormal chromosomes. Note in the interphase nuclei, the chromatin bridges, indicating chromosomal abnormalities that prevented a normal segregation.

These findings suggest the persistence of a population of hematopoietic stem cells with altered karyotype system due to the stress of the anticancer therapy stress that maintained their heterogeneous karyotype for up to 17 years after the treatment in 13/20 survivors. It is important to note that in these cells with heterogeneous genomes, cells of a clone do not necessarily show a CCA, due to the complex genome system generating daughter cells that may carry different NCCA resulting in highly dynamic karyotypes (Figure 3) [17]. This population of cells within each patient, is a source of conserved karyotypic heterogeneity that could eventually become a more stable population that acquire CCA and start a pathway towards the progression of cancer. These patients, and especially the one who presented with the clonal deletion, must be carefully followed in the oncology service because may belong to the 10-20% of HL survivors who develop a second cancer.

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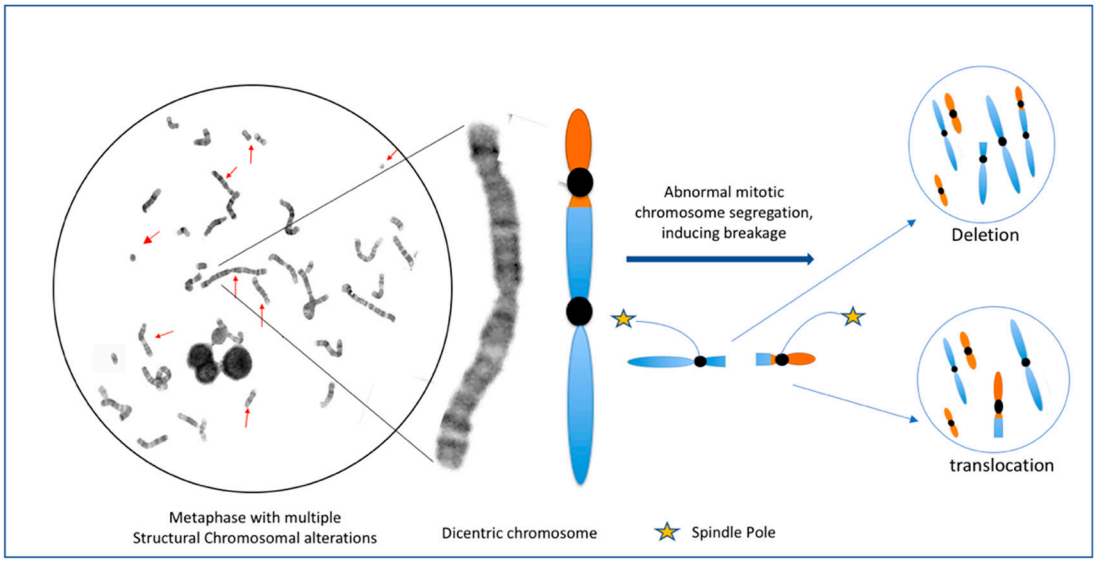

Figure 3. Metaphase of a lymphocyte from a survivor of HL after 13 years post MOPP treatment, with multiple structural NCCA [17]. Note that the possible daughter cells emerge with different alterations such as deletion or translocation, as a result of erroneous mitotic segregation of only one aberrant chromosome. Even when the daughter cells do not share the same structural aberration between them or with the progenitor cell, they may be clonal cells with multiple NCCA and without CCA.

Currently, ABVD CT is extensively used with or without RT as anticancer treatment for HL patients, because is considered less cytotoxic and genotoxic than MOPP, due to its lower content of alkylating agents and good preservation of sperm production. However, in a longitudinal study with HL patients treated with ABVD/RT, Ramos *et al*. [32] found that both NCCA and chaotic karyotypes were induced by the stress of this CT and RT in somatic cells, and the damage persisted at least until one year later [32]. The study was performed using multicolor fluorescence in situ hybridization (M-FISH) in metaphases of peripheral blood lymphocytes from patients diagnosed with HL, with sampling times before-treatment, during treatment (between second and third cycle of ABVD) and after-treatment, one year after the ABVD/RT. The analysis of 50-100 metaphases with M-FISH showed that NCCA consisted of both numerical and structural alterations, with structural NCCA being the most frequent type of chromosomal aberrations.

CIN was found in all patients, represented by NCCA involving both numerical and structural abnormalities, however, the highest frequency of damage was structural including simple and complex translocations. In addition, chromosomal chaos was observed one year after-treatment indicating that new aberrations were continuously produced in 4 out of 5 patients and only in one patient NCCA diminished after one year of treatment. Multiple translocations were found in the same cell, in addition to numerical NCCAs. After one year of the anticancer stress, samples presented with a 40-fold increase ($P < 0.0001$; one-tailed Fisher's exact test) in total abnormalities per cell (0.96 ab/ cell) with respect to control samples (0.024 ab/ cell). Whereas during-treatment and before-treatment samples showed a nine-fold and four-fold increase ($P < 0.0001$; one-tailed Fisher's exact test), respectively. The percentage of cells with NCCA was very high in 4 out of 5 HL survivors, ranging between 17.7-39.1% of abnormal cells with 1-56 abnormalities per cell. It is important to note that before-treatment the abnormal cells only presented with 1-5 NCCA / cell, during-treatment cells presented with 1-20 NCCA / cell and after-treatment aberrant cells presented with 1-56 NCCA/cell, including genomic chaos (Figure 4). CIN and genomic chaos had been referred to as characteristics of cancer cells, however, in this *in vivo* study, the cells are peripheral blood lymphocytes stimulated with M-phytohemagglutinin, from patients with no evidence of hematological malignancy or relapse of the original cancer [32].

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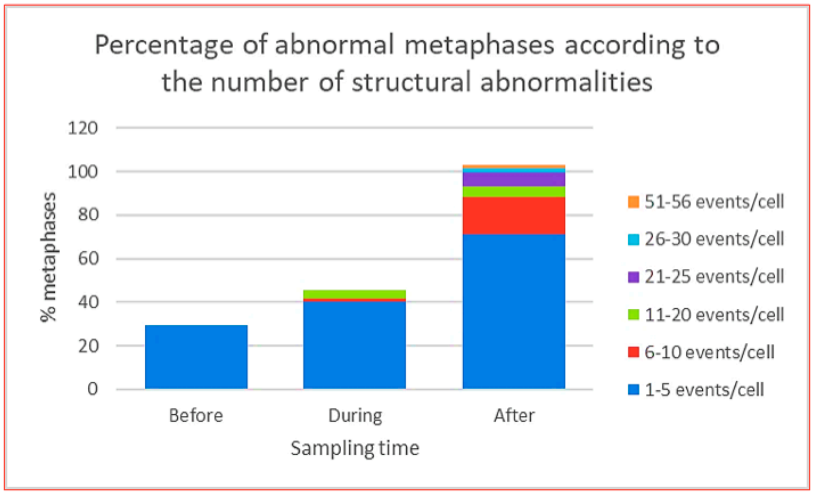

Figure 4. Total population of abnormal metaphases in lymphocytes from HL patients at each indicated sampling time, before, during and after the stress of CT ABVD / RT. The graph shows the percentage of abnormal metaphases according to the number of structural abnormalities per cell [32].

The study of Stephens *et al*. in 2011 [41] showed clearly that chromothripsis is present in at least 2%–3% of all cancers and in 2014, Liu *et al*. [31] monitored, in an experimental system consisting of four cell lines, the process of generating genomic chaos, demonstrating that CT agents, are able to induce karyotypic heterogeneity in a cell population *in vitro,* presenting with NCCA and diverse types of damage such as that associated with genomic chaos. This valuable information from *in vitro* studies or directly in tissue from tumors [31,41], strongly suggested that karyotypic heterogeneity could be produced by anticancer treatment *in vivo*, in non-cancerous cells, and that the population with karyotypic diversity could be the substrate for the evolution toward a new cancer related to treatment, which occur in a high proportion of patients [20]. The results of Ramos *et al*. [32] showed in vivo that a fraction of normal hematopoietic cells from HL patients respond to the CT/RT stress with a high proportion of NCCA leading to a heterogeneous cell population with complex karyotypes, which is continuously producing mature lymphocytes with NCCA, and demonstrated that diverse karyotypic systems are induced by anticancer treatment, as previously suggested by several authors [21,30,31] (Figure 5).

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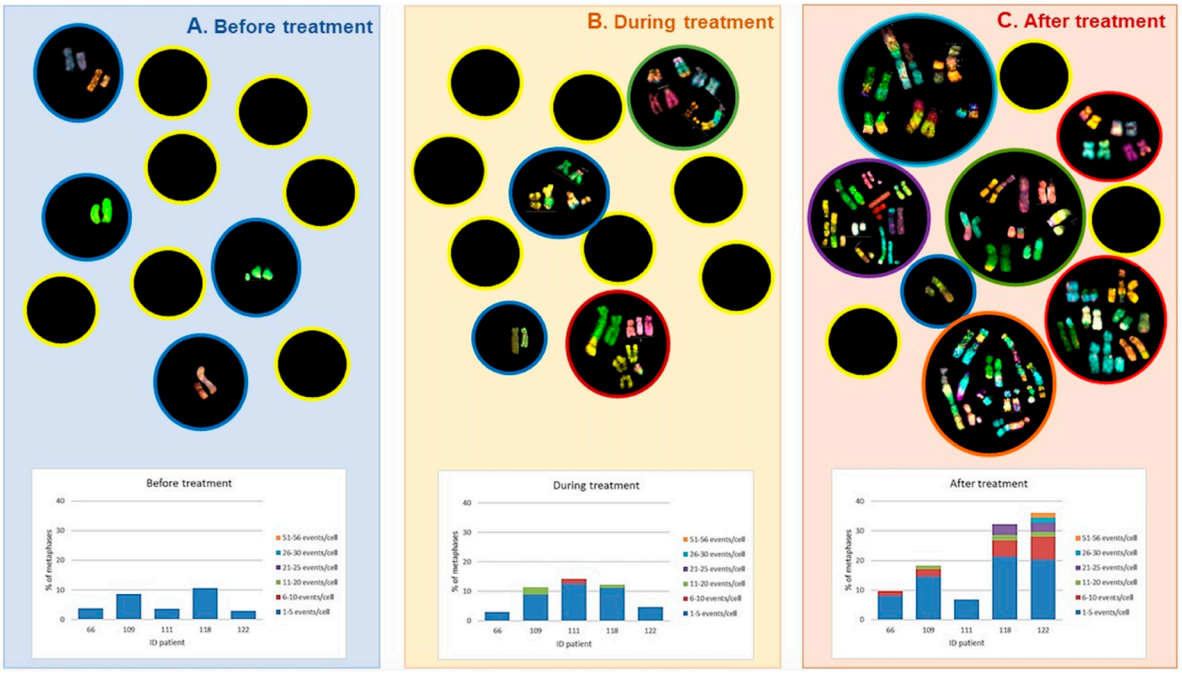

Figure 5. Upper part: M-FISH analysis of peripheral blood lymphocytes in samples from HL patients. Sampling was before, during and one year after completion of ABVD/radiotherapy treatment. Black circles represent cells: cells with yellow outline represent cells with normal karyotypes; cells with colored outlines represent cells with abnormal karyotypes. The graphs in the lower part show the distribution of the abnormal cells according to the number of chromosomal aberrations, for each patient. A. Before treatment, show alterations in less number and less complexity. B. During treatment, the number of cells with more damage increases even with complex karyotypes. C. After treatment, up to 60% of cells with chromosomal alterations were observed, and with the highest number of aberrations per cell, including chaotic karyotypes [32].

6. Impact of anticancer therapy on fertility and germ cell genotoxicity in patients with Hodgkin lymphoma

In the past 100 years, semen quality has been declining all around the world. In recent years, in several studies, a 30 – 40 % reduction in the sperm count in healthy men has been reported in several studies (reviewed in [42]). One of the possible causes of this, is the use of drugs that impair semen quality. It has been proven that individual chemicals, including those commonly used in CT, induce adverse effects on the quantity and quality of sperm; however, we know very little about the effects of complete CT / RT regimens on the risks of inducing chromosomal abnormalities in the sperm from treated patients [43].

In HL patients, as in other types of cancer, survival is improving as the therapies do, but long-term adverse consequences, including a strong effect on germ cells have been observed. Alkylating agents such as procarbazine are highly toxic to the testis, causing depletion of the germinal epithelium and aplasia of germinal cells [44,45]. Most patients with HL, have poor semen quality following treatment and a low sperm density that is presented as oligospermia and permanent or transient azoospermia [46,47].

Male HL patients who received MOPP as antineoplastic treatment with deleterious effects on spermatogenesis [15] show a significant decrease in semen quality; sperm count is affected, producing azoospermia or severe oligospermia even 22 years after the end of the treatment. Multiple studies have shown that MOPP, COPP, BEACOPP and ABVD treatments in patients with HL are genotoxic and induce azoospermia at different levels. The most aggressive treatment is

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MOPP since 90 to 100% of survivors have azoospermia [8,45,48–50]. In contrast, ABVD is less toxic causing transient azoospermia in one-third of patients, and most of them recover sperm production [49–51](Table 3).

Table 3. Consequences of anticancer treatment on the sperm count in HL patients

Anticancer treatment	Pre-treatment	Post-treatment*			Reference
	Normospermia (% of patients)	Normospermia (% of patients)	Oligospermia (% of patients)	Azoospermia (% of patients)	
MOPP	100	28	24	48	Da Cunha *et al.*, 1984 [45]
MOPP	100	3	0	97	Viviani *et al*., 198 [8]
MOPP	84	0	62	38	Meistrich *et al*., 1997 [48]
MOPP	100	20	35	45	Sánchez *et al*., 2008 [52]
MOPP	100	NA	NA	75	Bujan *et al*., 2014 [49]
ABVD	100	46	21	33	Viviani *et al*., 1985 [8]
ABVD	100	80	15	5	Sánchez *et al*., 2008 [52]
ABVD	100	100	0	0	Bujan *et al*., 2014 [49]
NOVP	100	50	50	0	Frias *et al*., 2003 [53]

NA=Not available. *The post-treatment time used in the studies was variable, from one month to 23 years.

The studies on genotoxicity in female germ cells are scarce due to the enormous difficulty of obtaining them in sufficient quantity and quality to perform the analysis. However, there are studies investigating cytotoxicity and fertility. Ovary is not a tissue with great proliferative capacity, it has a fixed number of germ cells produced during fetal life, and they complete their meiotic divisions during puberty, so they are more resistant than the testis to RT [54]. However alkylating agents are very toxic to the female reproductive organ, causing oocyte destruction and follicular depletion leading to ovarian failure and irreversible amenorrhea in a dose-dependent manner. Surviving HL women develop early menopause and absence of pregnancy [55]. MOPP, BEACOP and CHOP (Cyclophosphamide; Doxorubicin, Vincristine (Oncovin) and Prednisone) therapies cause infertility, ovarian failure and loss of gonadal function [56]. On the other hand, in women treated with CT without alkylating agents, ovarian failure is rare [57,58]. Female survivors of cancer who maintain fertility are at increased risk of miscarriage and/or premature birth and therefore require counseling and pre-conception evaluation by treating physicians [59].

The severe reduction of germ cells after CT/RT in HL patients is related with the genotoxicity of the drugs and radiation, which can directly induce DNA damage. The cell response to DNA damage requires DNA repair and, if not successful, cell death. The genotoxicity of CT treatments in germ

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cells from HL patients has been studied by several groups utilizing diverse methodology. Brandriff *et al*. [60], were the first to report the genotoxic effect induced by MOPP with and without RT in spermatozoa of HL patients. Using the technique of fusion of human spermatozoa with hamster eggs, to obtain and analyze chromosomes, they showed that between 3 to 20 years after receiving CT, sperm presented with 2% of numerical and 7% of structural chromosomal alterations indicating that the chemical agents were capable of damaging the germ stem cells of HL patients. The authors reported that damage "appeared to be not specific for chromosome pairs or regions to be involved in the structural exchanges", which may be interpreted as NCCA.

Sperm of HL patients treated with NOVP CT were studied with FISH for chromosomes X, Y, 8 [61] and X, Y, 18, 21 [53], in samples obtained before, during and after CT. A transient increase in the frequency of numerical chromosomal alterations was found, with a highest increase during treatment, which decreased three months after treatment.

ABVD treatment also produced a transient high frequency of chromosomal alterations in spermatozoa of HL patients that decreased 3-18 months later [50,62]. In an interesting study, Patassini *et al*. [63], analyzed the entire genome of 130 single sperms from three HL patients, at the end of three months of ABVD CT using aCGH (array Comparative Genome Hybridization) technique and found that 24% of the sperm carried numerical and structural chromosomal alterations. Specifically: 31 abnormal sperm presented with sex chromosome aneuploidies; 4/131 sperm with XY disomy; 3/131 sperm with XX disomy; 1/131 sperm with sex chromosomes nullisomy; and 23/131 sperm showed gains and losses in different regions of different chromosomes or complex alterations. According with the criteria for determining the type of chromosomal damage, Patassini *et al*., observed NCCA in these patients [63] (Table 4).

There have been several groups that have studied the genotoxic effect of anticancer therapy in germ cells, and based on to the results presented, it can be concluded that: 1) the treatment with MOPP induces long term genotoxic effects, while the treatment with ABVD induces transient genotoxic effects; 2) to observe the type of damage indicating NCCA and genomic chaos, it is necessary to carry out studies that evaluate, either cytogenetically or the molecularly, the whole genome, since the methodologies that use specific FISH probes generate information primarily associated with CCA.

Table 4. Studies on the Genotoxic Effect of anticancer treatment in HL Patients

Anticancer Therapy	Chromosomal damage	Reference (Technique)
	Lymphocytes	
MOPP/RT 6 cycles	*Chromosomal translocations, NCCA (Persistent up to 24 years)	Smith *et al.*, 1992 [35] (FISH, painting of chromosome 4)
MOPP/ABV 6-9 cycles	*NCCA structural. Chromosome breaks, acentric fragments, dicentrics and micronucleus (Persistent at six months)	Bilban-Jakopin and Bilban, 2001 [14] (Non-banded chromosomes)
BEACCOP, EBVP, ABVD,	*NCCA structural (Persistent up to 2 years)	M'Kacher *et al.*, 2003 [37]

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MOPP/ABV RT (combination not specified) 6 cycles		(FISH Painting of chromosomes 1, 3, and 4)
MOPP/RT 2-9 cycles	NCCA numerical and structural Persistent (up to 17 years)	Salas *et al*., 2012 [17] (G-Banding Chromosomes)
ABVD/RT 6-8 cycles	NCCA numerical and structural including genomic chaos (Persistent at 1 year)	Ramos *et al*., 2018 [32] (M-FISH)
	Spermatozoa	
MOPP/RT 2-6 cycles	*Numerical and structural NCCA (Persistent up to 20 years)	Brandriff ***et al***.,1994 [60](Non-banded chromosomes)
CHOP/MOPP/ABV 4-7cycles	Hyperhaploidy, disomy and diploidy (Persistent; decrease at 2 years)	Martínez *et al*, 2017 [50] (FISH, specific probes)
NOVP 3 cycles	Disomies, diploidies and complex genotypes involving the X, Y and 8 chromosomes (Transient; decrease at 3 months)	Robbins *et al*., 1997 [61] (FISH, specific probes)
NOVP 3 cycles	Disomies, diploidies and complex genotypes involving the X, Y and 18 and 21 chromosomes (Transient; decrease at 3 months)	Frias *et al*., 2003 [53] (FISH, specific probes)
ABVD/RT 4-8 cycles	Disomy XY, XX, Nullisomy 13 and 21 (Transient; decrease at 18 months)	Tempest *et al*., 2008 [62] (FISH, specific probes)
ABVD (number of cycles non specified)	Disomies XY, XX, Sex chromosome nullisomy, loss and/or gain of part of chromosomes and complex alterations	Patassini *et al*., 2013 [63] (microarrays aCGH)
ABVD/RT 4-7 cycles	Hiperhaploidy, disomy and diploidy (Transient; decrease at 3 months)	Martínez *et al*, 2017 [50] (FISH, specific probes)

*Authors describe chromosomal abnormalities that are compatible with NCCA, however they do not call it NCCA.

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It is important to highlight that MOPP contains procarbazine, which is one of the few chemicals that has been shown to affect germline stem cells [15], while other treatments such as ABVD do not contain agents that induce damage in them. This implies that the fraction of stem cell spermatogonia that had sustained genomic damage and survived MOPP therapy, can continuously generate sperm with genomic damage, and thus, HL patients treated with MOPP would have a long-lasting increase in sperm carrying genomic damage. Germline stem cell killing would also result in greatly reduced sperm count and azoospermia until the surviving stem cells begin cycling again. On the other hand, ABVD targets spermatocytes that would also generate sperm with genomic damage. However, once these sperm have been ejaculated there would be no 'record' of the exposure making the damage transient because germline stem cells are not affected. In addition, spermatocyte killing would result in a transient reduction in sperm count that would be quickly replenished by the unaffected stem cells.

7. Discussion

The treatment used for cancer patients, specifically HL, includes a series of drugs that have been shown to be cytotoxic and genotoxic and targeting dividing cells both in *in vitro* systems or in animal models. This strategy has been very efficient in eliminating cancer cells that are in continuous proliferation. However, because these compounds do not target cancer cells specifically, patients who survive cancer have a large number of non-cancerous cells in their body that were also affected by anticancer therapy. As a result of this stress, 10-20% of the population of HL survivors develop a new secondary cancer associated to treatment, among other secondary consequences. Studies performed in HL patients before and after treatment, indicate that the non-cancerous population of cells that managed to survive, have a variable number of cells highly affected at the genome level, due to the stress they suffered. Some of these cells show genomes with CIN, showing a high number of chromosomal alterations that when analyzed at the whole genome level by cytogenetics or molecular methodologies, are detected as having NCCA and genomic chaos, both in somatic and germ cells that can persist for a long period of time up to 24 years post-treatment.

Analyzing the results of several studies (Table 4), it can be observed that the anticancer treatments used in patients with HL affect hematopoietic stem cells, so that 1-24 years after treatment, cells with NCCA and even chaotic karyotypes can still be found. In germ cells, however, studies in sperm show that depending on the treatment used, the genotoxic damage may be transient or permanent. When the CT used was MOPP, germ cells showed a long-term effect and NCCA was found in HL survivors up to 20 years post-treatment, whereas when the CT did not include procarbazine, the damage was transient indicating that the stem cells had not been affected. All together, these studies show that the stress of anticancer treatment may have different effects depending on the type of stressor agent and on the type of cells.

Regarding the type of damage that has been found in HL patients and survivors, in all cases, the classic karyotypic damage has been detected, such as numerical alterations and structural alterations including deletions, duplications, translocations, dicentrics, rings, etc. However, observations primarily by Heng and his group [27] have shown that there is a great diversity of genomic alterations such as chromosomal fragmentation, asynchronous chromosomal condensation, abnormal interphasic figures, chromatin bridges etc., that have not been monitored and that represent non-classical genome damage induced by anticancer stress.

It is possible that not quantifying this type of alterations, may be the reason for some unexpected data in the study conducted by Ramos *et al*. [32], where a smaller number of classical cytogenetic alterations were found during-treatment, as compared with post-treatment samples. During-treatment samples represent the moment when all the cells of the whole organism were under stress, and it is during this moment when according with the results of Liu *et al*. [31], the non-classical genomic damage could be present in a significantly high proportion, and a fraction of the highly re-arranged and chaotic karyotypes could be eliminated. Liu *et al*. [31], found that when treating cells with chemotherapeutic agents such as those used in these patients, doxorubicin (Adriamycin) and

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alkylating agents, although there is a high frequency of cell death during stress, some of the chaotic genomes can survive and continue to change until the surviving genomes are selected. In fact, the observations made by Ramos *et al*. [32], and Salas *et al*. [17], resemble the pattern proposed by the *in vitro* experiments of Liu *et al*. [31] in 2014:

1) The stress of the anticancer treatment induced cell death of the cancerous cells, the treatment is successful to eliminate the cancer. However, genotoxicity and cell death are induced also in non-cancerous cells, such as hematopoietic cells; this process can generate a large number of cells with chromosomal fragmentation and some of them can survive.

2) The large number of DSBs induced by the anticancer treatment must be repaired by homologous recombination, which is error-free and acts only in the post-synthetic phases late-S and G2; this repair allows a proportion of the cells to repair the DSBs without generating karyotypic changes, although they can carry point mutations. However, an important repair mechanism for these lesions acting throughout the cell cycle is the non-homologous recombination or NHEJ, both classical or alternative route, and other similar mechanisms, involving abnormal replication [64], that can generate a random rejoining of the chromosomal fragments, producing a karyotypically heterogeneous cell population, with large CIN, leading the formation of highly re-arranged genomes and genomic chaos.

It is important to consider that the studies carried out on HL patients [17,32,60], have not documented the phase of C-frag that Liu *et al*. [31], considers a precursor of CIN and genomic chaos. Nevertheless, the finding of karyotypes with a large amount of complex rearrangements and chromoplexy show that there was fragmentation prior to the reshuffling of elements and that multiple DSBs must co-exist to be able to form chromoplexy and the observed complex rearrangements (Figures 1,2,5).

3) During the stress, most of the cells with chaotic genomes tend to die, which may partially explain the low frequency of cells with high NCCA in during-treatment samples from patients treated with ABVD [32]. However, the surviving cells generated daughter cells with inherited NCCA (Figure 3), preserving a population with genomic heterogeneity, since in samples post-treatment, one year or more, the alterations observed were NCCA and genomic chaos (Figures 2 and 5). This diversity of genomes is the substrate for the natural selection (punctuated phase of cancer evolution) and over time can generate a clone of cells with more stable karyotypes and in some cases CCA [21,26,31,33]. In the study of Ramos *et al*. [32] , one year after ABVD treatment, the surviving cells from HL patients presented NCCA but not CCAs [32], while in the study of Salas *et al*. [17], only in one HL survivor a CCA that involved alteration of chromosome 17p was found, 13 years after the therapy [17]. These data indicate that *in vivo*, the selection of more stable genomes, with CCA, and possible mutational and epigenetic changes, facilitating the evolution toward a new cancer (Stepwise phase of cancer evolution) may take several years [26,33,65]. This agrees with the information that indicates that second cancer related to therapy in HL survivors appear in approximately 10 years after treatment. These cancer, leukemia or solid tumors, present with NCCA, CCA and accumulation of somatic mutations directly associated with anticancer treatment [20,66].

Finally, the behavior of somatic cells is different than that of germ cells in HL patients; hematopoietic stem cells may retain for long time (up to 24 years) a population of cells with a high diversity of karyotypes, NCCA and genomic chaos after several types of CT, while germ stem cells only present long term NCCA after treatment that includes procarbazine (Table 4). Also, the consequences of this long term CIN are very different depending on the cell type; hematopoietic stem cells with NCCA may evolve toward a second cancer related to therapy, while germ stem cells with NCCA tend to disappear leading to azoospermia or oligospermia. According to Heng *et al*. [21], meiosis acts as a filter of genomic chaos because the reshuffling of segments of chromosomes makes the zygotene phase almost impossible, and most of the cells that survived to the CT stress, will die during meiosis. If stem cell spermatogonia that were exposed to MOPP are eliminated by meiosis, then, a majority of these HL survivors are azoospermic or oligospermic (Table 3), preventing the karyotypic heterogeneity at the organism level. Meanwhile, somatic hematopoietic cells do not divide

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by meiosis, and thus they preserve NCCA and may evolve toward a clone with selective advantage and cancer.

The data presented here resemble the results that Liu *et al*. [31] obtained *in vitro* and confirm that in HL patients, genomic chaos is generated by the stress of anticancer therapy, that a population of hematopoietic stem cells are preserved with great karyotypic heterogeneity and could be in the punctuated phase of evolution toward a second cancer. The fact that 80-90% of surviving HL patients do not develop a second cancer indicates that the environment represented by the whole organism has barriers that are insuperable for most cell populations with NCCA that could evolve towards cancer, but a 10-20 % manages to overcome these barriers, showing that evolution acts at the cellular level and in any environment.

It is important to know and understand the mechanisms that lead to the morbidity and mortality that cancer survivors present, since only in this way can new detection and treatment strategies be integrated for managing the secondary consequences of anticancer treatment in HL patients.

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