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Chimerism Analysis for Clinicians: A Review of the Literature and Worldwide Practices

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Abstract

This review highlights literature pertinent to chimerism analysis in the context of hematopoietic cell transplantation (HCT). We also conducted a survey of testing practices of program members of CIBMTR worldwide. Questions included testing methods, time points, specimen type, cell lineage tested and testing indications. Recent literature suggests that detection of low level mixed chimerism has a clinical utility in predicting relapse. There is also increasing recognition of HLA loss relapse to potentially guide rescue decisions in cases of relapse. These developments coincide with wider access to high sensitivity next generation sequencing (NGS) in clinical laboratories. Our survey revealed a heterogeneity in practices as well as in findings and conclusions of published studies. Although the most commonly used method is STR, studies support more sensitive methods such as NGS, especially for predicting relapse. There is no conclusive evidence to support testing chimerism in BM over PB, particularly when using a high sensitivity testing method. Periodic monitoring of chimerism especially in diagnoses with a high risk of relapse is advantageous. Lineage specific chimerism is more sensitive than whole blood in predicting impending relapse. Further studies that critically assess how to utilize chimerism testing results will inform evidence based clinical management decisions.

Introduction

Hematopoietic cell transplantation (HCT) is the only curative treatment for a variety of hematological malignancies and nonmalignant disorders. Major barriers for HCT success include failure of engraftment and relapse. Longitudinal monitoring of donor chimerism

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MA conceived the idea and MA and AGB searched and analyzed the literature and wrote the paper

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after HCT provides a means for monitoring engraftment as well as early detection of relapse (1, 2). Engraftment monitoring is particularly more critical with lower intensity preparative regimens, T cell depleted platforms (either CD34 selection or in vivo T cell depletion including post-transplant Cyclophosphamide, PTCy) and inadequately dosed grafts such as low dose cord blood. On the other hand early detection of relapse could guide the decision to initiate donor lymphocyte infusion (DLI) or a second HCT. In addition, vigilant post-transplant chimerism monitoring, particularly using high sensitivity chimerism testing methods and testing lineage specific chimerism, is gaining increased attention in cases of hematological malignancies associated with high risk of relapse (3–5). The technological evolution in chimerism testing methods has included erythrocyte phenotyping, cytogenetic analysis, fluorescent in situ hybridization (FISH), restriction fragment length polymorphism, DNA fragment analysis (variable number tandem repeats, VNTR and short tandem repeats, STR), real-time quantitative PCR (qPCR), digital droplet PCR (dPCR) and most recently, next generation sequencing (NGS) (6, 7). Each of these methods has its advantages and limitations. Currently there are no established guidelines for chimerism analysis in terms of clinical indications, at what timepoints or cell lineages to test. The purpose of this article is to present the results of survey on chimerism testing practices worldwide and summarize pertinent published literature as a preliminary step to establishing practice guidelines.

Methods:

Study Design

In November 2019, a web-based survey was distributed by email to HCT program director members (n=525) of the Center for International Blood and Marrow Transplant Research (CIBMTR). The survey (Supplemental Table S1) was developed, and data were collected using the web-based application Microsoft Forms (Microsoft Corporation, Redmond, WA). The results were exported and analyzed in Microsoft Excel (Microsoft Corporation, Redmond, WA). The survey recipients were provided a cover email invitation stating that by responding to the survey they agreed to participate in this study. The study was approved by Baylor Scott & White Health Research Institute Institutional Review Board (IRB# 019–291).

The survey included questions related to trends in chimerism testing after HCT. The survey focused on methodological and clinical utility considerations. Test utilization factors included time points of sample collection, specimen type and cell lineage tested. The clinical utility considerations included indication for testing. The survey also included basic descriptors for the programs to which survey respondents belong such as geographical area of practice, size and type of allogeneic HCT practices. Descriptive statistics were used to define the study population and the recorded responses.

Results:

Background of participants

Survey responses were received from 108 programs (21%). The demographic questions of the survey allowed characterization of the respondent programs based on geography,

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transplant volume, patient population and types of transplants performed. Our participant group represented a broad array of program characteristics (Table 1).

The participants spanned a wide geographic distribution. Approximately half of respondents (48%) were based in North America, mainly from the United States (n=45). The remaining North American centers included 5 from Canada and 2 from Mexico. Practitioners from Europe (n=17) and the UK (n= 3) comprised 20% of the survey respondents. The European programs were from France (n=5), Germany (n=3), Czech Republic (n=2), Spain (n=2), Switzerland (n=2), Belgium (n=1), Denmark (n=1), Italy (n=1). The remaining 36 participants represented HCT programs from South America, the Middle East, South Asia, Southeast Asia, Australia and New Zealand. Programs from South America included Brazil (n=8), Argentina (n=2) and Uruguay (n=1). Programs from the Middle East included Turkey (n=2), Saudi Arabia (n=2) and Israel (n=1). There were also programs from South Asia included India (n=6) and Pakistan (n=3) and Southeast Asia including Thailand (n=2) and Singapore (n=1). In addition, there were programs from Australia (n=4) and New Zealand (n=3). There were no responses from Africa.

A range of annual allotransplant volumes were represented among survey respondents, with the majority (51%) from HCT programs performing up to 50 transplants per year. Mid-range volume programs (50–100 HCT per year) comprised 36% of respondents. Larger programs, performing over 100 transplants per year, comprised 13% of respondents. Respondents included adult (50%), pediatric (22%) and combined adults/pediatrics (28%) HCT programs. In terms of type of transplants, all respondents performed HLA-identical transplants and over 90% performed matched unrelated donor (MUD). Alternative donor transplants included haploidentical, mismatched unrelated donor (MMUD) and umbilical cord blood (UCB) transplants in 99, 75 and 63% of the transplant centers, respectively. UCB transplants included single- or double-unit allografts.

Additionally, the survey participants were asked to describe the laboratory that performs chimerism testing in support of their HCT program. Most programs identified their laboratories as academic/university hospital-based (79%). The remaining participants identified the labs as non-academic, such as hospital-based labs (13%), reference/private labs (6%) or government labs (5%).

Testing Methods

STR analysis was the most common method reported by the survey participants (82%). Many also test by qPCR (23%). In terms of newer technologies, 7% of the programs reported using NGS methods for chimerism testing. Additionally, 4 respondents indicated that FISH was used in cases of sex mismatched HCT, with one participant commenting that FISH has a faster turnaround time at their institution. One participant also reported using VNTR analysis.

Testing Utilization

In terms of chimerism testing practices, respondents reported details regarding the post-transplantation testing time points and specimen type (bone marrow vs. peripheral blood and whole blood vs. lineage cell subsets).

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Figure 1 displays the responses related to the timepoints at which chimerism testing was performed after transplant. Most responses (94%) indicated testing chimerism at day 30, 73% indicated testing at day 90, 72% at day 180 and 63% at one year. Two respondents reported routinely testing earlier than day 30, one at day 15 and one at day 21, specifically in non-myeloablative (Non-MA) transplants. There were also a number of responses indicating day 60 testing (7%). Some participants indicated that routine chimerism testing was performed until complete donor chimerism was established (n=6). Two respondents specified routine chimerism testing for 2 years and two other respondents indicated yearly testing up to five years. A few respondents indicated that timing of chimerism testing was dependent on clinical status including WBC engraftment (n=2) and suspected relapse (n=1). Furthermore, 3 responses indicated that timing of testing differed by disease diagnosis without further clarification.

Regarding specimen source, practically all programs (99%) use peripheral blood (PB) with 89% indicating PB as the primary specimen type used in chimerism testing. Sixty nine percent of respondents also test bone marrow, of which 13% cited bone marrow as the primary specimen type tested. One participant also reported using tissue samples in cases involving post-transplant lymphoproliferative disorder (PTLD).

In terms of lineage specific testing, 75% of the responses indicated testing whole blood, 67% indicated T cells, 46% indicated myeloid cells, 18% indicated B cells, 14% indicated NK cells and 9% indicated CD34+ cells as shown in Figure 1.

Clinical Indications

All survey respondents reported engraftment monitoring as a primary indication for chimerism testing. Most programs also indicated using chimerism testing in immunotherapy planning such as DLI (85%) and in detecting impending relapse (69%). One respondent indicated that chimerism testing was used to identify donor-derived malignancy.

Participants were invited to leave additional comments regarding indications. Multiple respondents (n=4) indicated that chimerism testing strategies are disease-specific and/or dependent on conditioning regimen used for HCT. One participant specifically indicated that in their program, chimerism is not routinely performed in the setting of myeloablative (MA) conditioning. In the context of predicting impending relapse, two participants commented that the utility of chimerism testing is limited to cases in which a disease-specific marker (e.g. fusion protein transcript) is not available. One response indicated uncertainty about therapeutic intervention implications for decision making when mixed chimerism (MC) is detected. Additional comments included remarks regarding feasibility in terms of cost and technical considerations of lineage specific analysis.

HLA Loss Relapse

Vago *et al.* identified cases of post-haploidentical HCT relapse in which the patient-specific HLA alleles were undetectable (8, 9). They further demonstrated an underlying copy-neutral loss of heterozygosity of the short arm of chromosome 6 (6p) encompassing the HLA region through substitution for the “lost” haplotype of a corresponding region from the homologous chromosome, a phenomenon known as acquired partial uniparental disomy.

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Several questions in the survey focused on testing for HLA loss relapse, as previously described (9). However, only 40% of the respondents completed these questions and represented a variety of viewpoints and practices. Only 3 participants indicated that HLA loss testing was currently available at their institution. Utilization varied with one program indicating testing only for haploidentical transplants. Another reported testing on all patients transplanted for hematologic malignancies. Methods reported included commercial and in-house developed qPCR assays.

Most responses (n=32) indicated that they would use the test clinically, if it was available, particularly for haploidentical transplants. Several also indicated that they would use this test in unrelated donor (n=17) and cord blood (n=9) transplants as well. When asked to consider timepoints that they would test for HLA loss, responses varied but the majority reported that they would test for HLA loss when chimerism analysis showed autologous recovery (MC, increasing recipient chimerism). Additional responses included "at time of relapse", "with every chimerism test" and "unsure." The low response rate for HLA loss relapse testing may reflect unfamiliarity with this clinical context and highlights the need for developing guidance regarding the clinical utility of this testing modality.

As to how results would influence patient management, free-text responses generally fell into two categories: 1) guiding decisions for DLI or immunosuppression tapering or, 2) guiding second HCT and donor selection. Regarding cellular therapy decisions, respondents indicated that if HLA loss were detected, this would influence their decision not to give DLI, and may guide non-DLI relapse management strategies. In terms of decisions for second HCT, participants commented that HLA loss could be used to identify a patient requiring second HCT and may be used to select a donor with a 100% mismatch for the residual leukemia haplotype.

Although many participants indicated interest in the clinical utility of the assay, there were several respondents (n=7) that indicated they would not use this test. One participant commented that there is no clear evidence of clinical utility outside of haploidentical transplants.

Discussion and Review of the Literature

Two decades ago, the lack of "logical, consistent, and uniform set of recommendations" for chimerism analysis in clinical HCT prompted the National Marrow Donor Program of the US (NMDP) and the International Bone Marrow Transplant Registry (Currently CIBMTR) to sponsor a workshop focused on the use of chimerism analysis after allogeneic HCT (10). Twenty years later and despite significant technological advances in testing methods, we still lack widely accepted practice guidelines regarding chimerism analysis testing, as evidenced by the notable heterogeneity of practices worldwide revealed by this survey. Perhaps that stems from the paucity of studies formally evaluating technical and clinical attributes of this diagnostic and prognostic tool. Critical considerations for integrating this test into clinical care include time points at which chimerism is performed, whether and which lineage specific chimerism to analyze, whether to test PB or BM, whether to test for HLA loss relapse and analytical sensitivity needed for the clinical setting. To make crucial treatment

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decisions, clear guidelines are needed to inform which chimerism analysis is most relevant and at what result threshold to take action.

Our survey of practices among transplant centers reporting to CIBMTR from different parts of the world indicated some general themes as well as notable differences in such practices. There was a reasonable representation of programs with varying attributes of programmatic backgrounds. Participants were from countries in North and South America, Western and Eastern Europe, United Kingdom, the Middle East, Asia, as well as Oceania. The lack of responses from African countries may reflect overall small number of HCT programs and/or low level of participation in international transplant registries such as CIBMTR. The majority of responses represented programs with academic affiliation, which may reflect the necessity of multi-disciplinary teams typically more readily available in academic institutions. However, government and private hospitals were also represented. Survey participants represented both adult and pediatric transplant programs. The majority of responding programs were medium size performing between 20–100 transplants a year. Responding programs represented those performing allotransplants using all types of donor sources including HLA identical, matched and mismatched unrelated as well as HLA haploidentical donor transplants. About half of the represented programs performed UCB (single and double) transplants. Results of this survey give an approximate representation of worldwide clinical practices related to chimerism testing following HCT. Here we discuss the findings of the survey results in light of the pertinent published literature. A challenge in reconciling consistency, or lack thereof, among different studies correlating chimerism test results to specific clinical outcomes is the remarkable heterogeneity in terms of graft source, conditioning regimens, diagnoses, number of cases, testing method, definition of MC, time points tested, specimens tested.

Testing Methods

The basic principle of chimerism testing is to identify genomic regions with enough diversity of polymorphism to distinguish donor versus recipient genetic origin in a given clinical specimen. The performance characteristics and availability of genetic markers to make this distinction are not universal among various test methods and are described elsewhere (11). The method used dictates the analytical sensitivity and availability of polymorphic markers to interrogate the genetic differences between the donor and recipient.

The vast majority of respondents reported using the semi-quantitative PCR amplification of genomic regions containing STRs. About a fifth of responses reported using qPCR and only a minority reported using recently introduced NGS based methods. Both qPCR and NGS rely on multiplex detection of single nucleotide polymorphisms (SNP) and/or genetic variation in the form of insertions or deletions (indel) polymorphism across multiple chromosomes (12–14). The reported proportions of method utilization are consistent with those reported in the published literature referring to chimerism analysis testing in the clinical context of HCT. Figure 2 summarizes the most significant differences among the three most commonly testing methods by the survey respondents (STR, qPCR, NGS). Three participants reported using FISH and no responses indicated using other approaches such as cytogenetics, red cell phenotyping, RFLP analysis or dPCR (15, 16).

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The number of polymorphic markers used for chimerism analysis ranges from 16 markers in most STR based tests to 39 markers in commercial qPCR-based methods and hundreds of markers simultaneously interrogated by NGS. The availability of commercial testing reagents to interrogate specific genomic regions such as the HLA region has been thus far limited to the qPCR platform(8).

STR based tests have been reported with a sensitivity limit ranging 1–6%. The sensitivity of this method is dependent on the percentage of donor cells present. If the donor cells are 95% to 99%, the assay sensitivity is 1%, whereas for 90% to 94% donor cells the sensitivity is 3%, and for 11% to 89% donor cells the sensitivity of the assay is 6% (17). The reported sensitivity of qPCR methods (0.01%) is orders of magnitude higher than STR, however, their precision (variation coefficient of 30–50%) is much lower than STR (1–5%)(12). Comparable sensitivity between NGS and qPCR has been reported previously in detecting MRD in B cell malignancies (18). However, NGS offers much higher precision than qPCR with CV < 2% at MC levels 5%. In the range of 0.1%–90%, NGS methodology is more accurate and more linear than qPCR and STR analysis (19).

Another high-sensitivity methodology that has been utilized for chimerism analysis is digital droplet PCR (dPCR). This method has been demonstrated to have the high sensitivity of a qPCR method, but with higher accuracy and reproducibility, due to the droplet technique whereby a DNA sample is partitioned into multiple nanoliter droplets, and in essence, separate reactions (20). This allows for end-point PCR detection and eliminates the need for calibration curves, and multiple replicates, typically required for qPCR. Several groups have reported on the analytical performance of dPCR chimerism analysis, including sensitivities from 0.01% to 0.1% (21–23). Specifically, compared to qPCR, dPCR has been demonstrated to be an effective alternative for chimerism analysis with high accuracy across the analytical measuring range from 0.05% to 100% (24–27). The limitations of this method include inaccuracies in the setting of high DNA input and limited availability of informative markers (bi-allelic, similar to qPCR). Despite, these drawbacks, the feasibility of this technique in the clinical diagnostics setting has been reported (16, 25, 28). However, the instrumentation is not yet as widely available as that of NGS, which are becoming commonplace in laboratories that are performing HLA typing for HCT donors and recipients.

Collectively, one can appreciate that STR is the most commonly used method, qPCR follows in popularity and is significantly more sensitive than STR but lacks precision whereas NGS is the least commonly used and combines the highest sensitivity and precision. In addition, NGS is being increasingly adopted by laboratories performing HLA typing for HCT donors and recipients which alleviates initial instrumentation cost of implementing NGS based chimerism testing (29, 30). It is essential to appreciate that the majority of chimerism analysis tests performed worldwide are laboratory developed tests, and the sensitivity limits of the same platform may vary significantly at different levels of chimerism detected, among different testing reagents and even between different laboratories using the same testing reagent. For example, NGS sensitivity has been reported at ranges of 0.0001–0.1% (31). Currently there is no consensus on how to define sensitivity which is often based on contrived chimeric samples of healthy subjects rather than real patient data. Consequently, acceptance criteria defined by individual testing laboratories may

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overestimate the sensitivity achievable in clinical patient samples. This often-unreported critical piece of information imparts an additional layer of complexity when attempting to reconcile clinical outcome correlations with chimerism analysis results at a specific time point, cell lineage or at a specific cutoff of MC percentage.

Time Points for Testing

Routine chimerism analysis after HCT is performed to determine donor engraftment status and guide potential prophylactic or salvage strategies such as rapid withdrawal of immunosuppression and/or DLI (32, 33). There are some similarities and differences in clinical practices revealed by the survey responses regarding the exact time points at which chimerism analysis is performed. The majority of respondent programs perform chimerism analysis by day 30 post-transplant with other common time points around 3-, 6- and 12-months post-transplant.

Recommendations from a workshop at the 2001 Tandem Meetings of the International Bone Marrow Transplant Registry (currently CIBMTR) and the American Society of Blood and Marrow Transplantation (currently the American Society of Transplantation and Cellular Therapy or ASTCT) suggested performing chimerism analysis testing at 2–4 week intervals until stable and sustained engraftment is achieved and at 1, 3, 6, and 12 months (10). More recently, another group reported performing serial analyses of chimerism weekly until day +200 and monthly thereafter, with the intent to treat patients showing MC with pre-emptive immunotherapy (IT) (34).

Achievement of early complete donor chimerism (CC) within 30 days after allogeneic HCT was associated with both lower probability of relapse and longer relapse free survival (RFS) but not with overall survival (OS) compared to those with late CC (35). When chimerism testing was performed at the time of neutrophil engraftment, on day +30, and 3, 9 and 12 months after transplantation, day +30 CC was the only time point associated with lower risk of relapse (32). Similarly, OS and RFS in patients experiencing CC was not statistically different from patients demonstrating MC at days 30, 60, and 90 following SCT (36). Another study reported that OS in pediatric patients who achieved CC on day +14 did not differ statistically from patients with MC on day + 14 (37). A retrospective study showed that CD3+ lineage MC at day 80 post-transplant was not associated with transplant outcomes after myeloablative HCT for ALL (38). A similar conclusion was reported by another group suggesting that routine testing of chimerism in BM and PB at 2 to 6 months after myeloablative HCT has limited prognostic utility but may be helpful in documenting engraftment in clinical trials (39). However, chimerism analysis methods used in many of the studies that concluded a lack of clinical utility of serial monitoring were a mix of STR and less sensitive methods such as VNTR which may have underestimated the proportion of patients with MC by misclassifying them as CC. In contrast, when qPCR chimerism analysis was performed it revealed that patients with chimerism >1% who received preemptive IT had significantly lower relapse rate than those who did not receive such therapy (2). In this study the median time to detecting MC was 4.5 months (range: 1–31 months) and the median time from MC detection to relapse was 1 month, suggesting a clinical utility of chimerism monitoring using a sensitive testing method up to at least 31 months.

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The duration of follow-up time for chimerism monitoring should be based on risk for relapse post-HCT (40). Although relapse is most common in the first 2 years, several groups reported significant cumulative incidence of relapse in patients beyond 2 years (41–44). Recommendations for scheduling routine chimerism analysis is not a one-size-fits-all. The frequency of testing and duration of monitoring should be a function of the risk of relapse. While most relapses occur in the first 2 years, in the light of documented relapse and relapse-related mortality in the first 5 years, frequent monitoring in the first 2 years is recommended; however, periodic monitoring up to five years in cases of increased risk with relapse is warranted.

Failed and delayed engraftment are serious concerns in CBT even when using double cords which renders chimerism analysis of particular clinical relevance in this clinical context. In the double cord transplant setting, dual chimerism was detected at day 21–28, with subsequent single chimerism in 97% of the cases by day +100 and beyond (45). In another CBT study, the donor unit with early (days +7 and +14) higher CD3+ T cell or CD33+ myeloid cell chimerism became the dominant engrafting unit (46). When chimerism was analyzed at day +21 in double unit CBT recipients, 100% DC results was associated with 98% engraftment at a median time to engraftment of 22 days. In comparison, recipients who were 90–99% DC at day +21 had 100% engraftment with a median of 29 days and of those with <90% DC, only 68% achieved engraftment at a median of 37 days.(47). In this study, day +21 chimerism analysis results predicted median time to CC and identified recipients at risk of delayed or failed engraftment.

Lineage-Specific vs. whole blood

The majority of respondents indicated testing whole blood (WB) or T cell lineage followed by myeloid cell chimerism. Fewer responders indicated analysis of chimerism in B cell and NK cell lineages and a minority indicated testing CD34+ cell chimerism. It is noteworthy that immune reconstitution of different cell lineages after HCT occurs at different time points, starting with neutrophils at 14–30 days (depending on type of the graft), followed by NK cells at 30–100 days, T cells at 100 days and lastly by B cells taking up to 1–2 years after HCT (48). Being mindful of differences in reconstitution rates is critical when interpreting lineage specific chimerism results and when monitoring engraftment of specific lineages. In addition, differences in conditioning regimen, graft source and processing affect chimerism, most easily demonstrated by lineage-specific analysis (49).Several groups have reported that detectable lineage-specific MC levels were associated with clinical outcomes (50–58).

Lineage-specific chimerism analysis is also suggested to have higher sensitivity compared to WB chimerism (10, 17). It is intuitive that MC occurring in only one lineage could be missed when chimerism testing is performed on WB where the MC in one lineage becomes diluted by CC in other lineages. An example is when MC of a low frequency cell population such as T cells following T cell depleted/reduced HCT are below the limit of detection in WB but would be detected in a T cell-enriched specimen.

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T Cells

T cell subset chimerism is commonly evaluated for monitoring patients following non-MA allogeneic HCT (10). A number of studies have shown that T cell MC is associated with relapse in several settings, including myeloid and lymphoid malignancies and with both MA and non-MA regimens (51, 54, 55, 59). In the haplo-cord HCT setting, persistent haplo-chimerism in the CD3+ lineage was associated with an increased rate of disease recurrence (60). In contrast, other groups have found no association between T cell MC and risk of relapse (38, 61–64). A recent retrospective study reported that in the setting of T-cell depleted grafts, T cell MC is the major contributor of MC that can be detected in WB samples, and was not associated with graft failure or relapse in a pediatric population undergoing transplant for malignant or nonmalignant diseases (49).

A common theme observed among different studies was the underappreciated inconsistency and often incomplete reporting of testing method sensitivity, the cutoff % defining MC and testing time points that were correlated with clinical outcomes. Thus, it is incredibly challenging to compare findings and conclusions across multiple studies. With these limitations in mind, T cells chimerism not only was reported to offer more sensitive detection of MC, but also as a cell population is present at sufficient frequency in PB to isolate at high purity and yield for performing chimerism testing (10). This may at least in part explain the predominance of testing T cell lineage among the majority of our survey respondents.

Myeloid cells

Mixed chimerism in the myeloid lineage defined mainly by CD33+ or CD15+ cells, has been examined in a few settings including CML, haplo-cords, and myelofibrosis and may have utility in patients for whom molecular and morphologic studies provide limited information (60, 65, 66). A retrospective analysis found that early CD33+ MC (days +30 & +60) did not impact RFS or OS (67). However, this report did not address whether clinical management decisions were made based on these results and might have contributed to comparable outcomes with patients who achieved CC. In addition, the combination of using high sensitivity qPCR method and a strict threshold of MC of <100% may have overrepresented the MC chimerism group compared to previous studies by including patients with negligible percentages of recipient chimerism that was inconsequential. The conflicting data highlight the need for carefully designed studies that address the heterogeneity of practices and clarify the potential utility of this test.

B Cells

Chimerism testing is performed infrequently on CD19+ cell lineage, and there are few reports in the literature to suggest a role for B cell chimerism analysis after allogeneic HCT (68–70). In a small study of B-ALL patients, Yang et al. reported that B cell chimerism was more sensitive for both graft rejection and relapse, compared to T and NK cell chimerism (69). In contrast, another study reported no significant correlations between B cell MC and an elevated risk of relapse (59). Taken together, while there is some suggestion of an association between B cell chimerism and relapse at least in B-ALL, larger studies are needed to further validate the potential utility of B cell chimerism analysis.

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NK Cells

There is a paucity of published studies that specifically investigate the predictive utility of NK chimerism, and the findings are inconsistent among different patient populations such as in MA vs. RIC. Breuer *et al.* reported that monitoring of T and NK-lineage chimerism can risk stratify patients for graft loss and suggested an algorithm for risk-based assessment at an early timepoint for proactive therapeutic intervention to stabilize the graft (53). Other studies reported no predictive value of MC including testing of T and NK cell lineages (38, 39, 59). The seemingly conflicting conclusions may relate to heterogeneity of studies including differences in conditioning intensity, frequency and timepoints of testing and MC cutoffs.

CD34+ Cells

Earlier studies suggested a comparable sensitivity and specificity of CD34+ lineage chimerism to WB chimerism as a predictor of impending relapse (71). However, more recent studies have reported that decreasing PB CD34+ donor chimerism was strongly associated with a higher cumulative incidence of relapse and lower RFS and OS (52, 59, 72). In these studies MC definitions ranged from any detectable recipient chimerism to DC <80% by STR methods. It's worth mentioning that in these studies, relapse in patients who did not show decreasing CD34+ donor chimerism was still considerable. Whether using more sensitive methods such as NGS or lower thresholds to define actionable MC would have identified MC in cases not detected by STR remains an open question for future studies.

Overall, these studies suggest that CD34+ cell subset chimerism is more sensitive in predicting relapse than WB chimerism. However, it remains unclear whether CD34+ chimerism is more predictive than the more technically feasible and commonly performed PB CD3+ lineage chimerism. Further investigation using highly sensitive chimerism methods are needed to establish the predictive utility of CD34+ cell chimerism.

Exploring the immunobiology of engraftment of different lineages may further elucidate these observations and clarify the role of lineage subset chimerism analysis in post-transplant monitoring for graft stability and risk of relapse.

Bone Marrow vs. Peripheral Blood

All responses indicated that chimerism analysis was performed primarily on PB and less commonly on BM. The vast majority of participants indicated using PB most of the time whereas two thirds indicated never or rarely using BM. Among the survey respondents who use bone marrow as a source more frequently than peripheral blood, 100% were using STR methodology exclusively.

Intuitively, the earliest evidence of graft failure or relapse would be identified by analyzing bone marrow, the "niche" of engraftment. The higher percentage of recipient DNA in BM compared to PB has been well documented in numerous studies (73–75). This simple fact renders the question of whether to use PB or BM for chimerism analysis inseparable from the sensitivity of the testing method. However, the more readily detectable recipient DNA in BM vs. PB does not unquestionably impart more clinical actionability on BM chimerism results. Almost two decades ago and based on published literature at the time,

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it was recommended that chimerism analysis performed on PB is more actionable than BM (10). In addition, the remarkable improvement in analytical sensitivity of newer methods for quantification such as NGS enabling detection of less than 0.1% chimerism in PB may reduce the need for performing BM chimerism (75).

There is mixed literature regarding the equivalency or lack thereof of chimerism testing in BM vs. PB. In one study, chimerism analysis was performed in patients who relapsed and those in stable remission using a sensitive qPCR method of both PB and BM (73). The study reported an increased recipient chimerism in BM samples defined as 0.5% observed in 90% of patients before relapse. The study also reported that in paired BM and PB samples collected at time of relapse, all BM samples had significantly higher recipient DNA % than PB samples. Although these results may imply a higher false negative rate in chimerism monitoring in PB, it is challenging to interpret without assessing the false positive rate of recipient DNA > 0.5 % in patients who did not experience relapse, which was not presented in this publication. Detection of MC in BM earlier and at a higher frequency than in PB does not necessarily impart higher diagnostic utility on BM chimerism. These early changes in chimerism detected only in BM may reflect dynamic changes that are potentially self-correcting and may not warrant therapeutic intervention. In a recent study of patients that showed CC by STR, qPCR revealed that 59% had 0.1–1% recipient DNA in BM while only 7% presented 0.1–1% recipient DNA in PB (76). Of the latter group one of 4 patients relapsed suggesting that the detection of recipient DNA in PB is much less frequent than in BM but may have superior clinical utility in early detection of disease relapse. In a prospective observational study of twenty patients with high/very high-risk AML (the KIM-PB prospective study), Gambacorta *et al* evaluated the performance of serial qPCR chimerism analysis in BM vs PB for the prediction of relapse (77). Paired BM and PB samples showed moderate correlation with higher recipient DNA % in BM compared to PB, regardless of relapse. In this study, the most predictive results of relapse were achieved in both PB and BM when considering only those samples with host chimerism increasing by > 19.4% of the value of the previous determination, and exceeding the threshold values of 0.13% for PB and 0.24% for BM. Using this model of calculation, increasing PB chimerism had a higher sensitivity and specificity for relapse than BM chimerism, although not statistically significant. The authors attributed this, in part, to more frequent sampling, which is feasible with the less invasive PB compared to BM.

PB chimerism is also more informative in the setting of non-MA transplants where distinguishing myeloid and lymphoid mixed/split chimerism may be more readily detectable in PB, where T cells represent a significantly higher percentage of total leukocytes than in BM (10). Even further, there is strong evidence that higher recipient DNA contribution is explained at least in part by BM samples containing stromal cells that may constitute a source of false positive MC results in BM (31, 78, 79). While stromal contamination may vary depending on variations in BM sampling technique, it is expected, with sequential chimerism testing, that a true increase in mixed chimerism would be detectable above background contamination. Other sources of nonhematopoietic cell contamination may include skin plug from phlebotomy sample collection, which may be more evident using a higher sensitivity method (80).

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Another consideration for performing chimerism on PB vs. BM is whether lineage specific chimerism is being tested, since it is inherently more sensitive than WB chimerism. In a study of pediatric AML, patients with MC defined as >1%, detection of MC in CD33+ and $CD34^+$ BM subsets was documented before identification of MC in the WB or the whole BM (mean: 32 days, range, 7–74 days) [81]. However, in this study no comparison was made between chimerism tested in the same lineages from BM vs. PB.

Taken together, in addition to BM collection being more invasive than PB, there appears to be no conclusive evidence to support a superior prognostic performance of chimerism studies on BM compared to PB. Further studies with sensitive chimerism testing methods that longitudinally compare recipient DNA percent detected in PB and BM in patients who relapsed as well as those in complete remission is warranted.

Clinical Utility

Engraftment Monitoring—All survey respondents reported that the primary clinical indication for chimerism testing was engraftment monitoring. It was also indicated that chimerism may not be routinely performed in the setting of MA conditioning. Studies have demonstrated that MA conditioning leads to consistent engraftment (82, 83). In combination with hematopoietic recovery (ANC, platelet count), chimerism testing is required to demonstrate that hematopoiesis is of donor origin as part of documenting allogeneic transplantation success (6, 10, 15).

Guiding Immunotherapy Management Decisions

The majority of survey respondents reported using chimerism analysis to guide decision making with regards to DLI and the prediction of disease relapse. Prediction or early detection of relapse may allow for more timely intervention, such as the administration of DLI and tapering of immunosuppression in an effort to elicit or enhance the graft versus leukemia (GVL) effect for pre-empting an overt relapse and improving prognosis.

Chimerism testing also detects the re-emergence of host hematopoiesis following allogeneic HCT engraftment. The clinical utility of MC has been investigated extensively. While once considered a harbinger of relapse, in certain settings, MC may simply represent autologous hematopoietic recovery and not necessarily relapse of malignancy. Early studies reported conflicting findings, with some studies showing correlation between increasing levels of MC with relapse of acute leukemia post-HCT while others did not demonstrate similar correlation (84–88). The discordant results may in part stem from using testing methods with varying sensitivity limits and variable monitoring frequency (84–87, 89–91). With advances in testing methods, it has been observed that MC is a more common state than previously thought and that the level of detectable MC is rather dynamic. After allo-HCT, MC undergoes continuous undulations which may not necessarily predict an impending relapse. Using qPCR, small increases in recipient cells (as small as 0.1%) can be detected; however, successive increases in MC (rather than a single timepoint) are predictive of relapse (92). More recently, in another large retrospective study of microchimerism kinetics, over 900 samples from 71 acute leukemia patients were tested by both STR and qPCR post allo-HCT. Sellmann *et al.* observed detectable MC (>= 0.1%) in 87% of the samples tested

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by qPCR vs. 10% of samples tested by STR (93). Instances of increasing MC that were not associated with relapse were most frequently seen in the context of viral infections. In terms of relapse prediction, they reported that qPCR excluded relapse with a high negative predictive value (98%) and predicted relapse more than one month prior to clinical relapse (median of 45 days) (in this study, relapse was defined according to the Cheson criteria: reappearance of leukemic blasts in PB or >5% in BM (94)). Uniquely, Sellmann *et al.* used a kinetic parameter (termed an increment factor) that accounted for the rate of increasing MC and duration of steady increases, in addition to absolute MC levels. After day 150, absolute MC >0.1% and increment factor >1.020 were significantly associated with relapse. The combination of the two parameters improved prediction of relapse, compared to either parameter alone. Including duration of increasing MC (t) as a parameter further improved the performance at these optimal cutoffs although the positive predictive value did not exceed 72%. However, the trade-off/compromise for increased specificity and positive predictive value is a decrease in time to relapse (Table 2, modified from Sellmann et al), from a median of 38 and 45 days (for absolute MC >0.1% and ICF >1.020, respectively) to 17 days.

This study confirmed that a kinetic approach to chimerism analysis predicts relapse with a higher specificity and PPV than using absolute MC levels only at individual time points.

In a recent letter to the editor, Mountjoy and colleagues questioned the utility of continued early chimerism testing based on a retrospective analysis of single center data showing that neither T cell nor myeloid cell chimerism at day 30 or day 60 had statistically significant impact on OS (67). Furthermore, the authors specifically examined the subgroup of patients transplanted for myeloid malignancies and also found that early CD33 MC did not impact RFS. The authors concluded that early lineage specific MC was not associated with OS or RFS and suggested that it would not add to molecular and flow cytometric analyses done to monitor MRD for early relapse detection. In contrast to previous studies, the combination of using high sensitivity qPCR method and strict threshold of MC of <100% may have overrepresented the MC chimerism group by including patients with infinitely negligible percentages of recipient chimerism, which is potentially non-consequential, among the MC group. In addition, the retrospective nature of the study does not preclude the possibility that management decisions were made based on these chimerism results and may have contributed to the observed lack of correlation among outcomes and chimerism state. Direct comparisons to other studies with less sensitive methodology must be interpreted with caution.

The 3-year EFS of childhood ALL patients with increasing MC without IT was reported in one study at 0% (85). IT with rapid withdrawal of immunosuppression and DLI based solely on persistent MC has been attempted in pediatric hematologic malignancies (95). In this study, IT was initiated in cases with persistent 1% of donor cells in any of the tested PB or BM lineages (CD3+, CD14/15+, CD19+ from PB/BM; CD33+, CD34+ from BM) at day +30 and confirmed on 2 subsequent tests done 2 weeks apart in spite of 100% chimerism in WB. Patients with any evidence of acute GVHD or peri-engraftment syndrome and patients with > 1% leukemic blasts on the first post-transplantation disease evaluation were excluded from this intervention. In this series, there was no significant difference

in 2-year EFS among patients who received chimerism based IT (pre-emptive DLI) and patients who achieved CC spontaneously. It is noteworthy that 50% of all relapses in the IT group occurred later than 2 years post-transplantation, whereas no late relapses were observed in the spontaneous CC group, leading to worse EFS in the IT group at the mean study follow-up of 42 months. Compared to patients who achieved spontaneous CC, there was no increased incidence of GVHD. A longer follow up of a subgroup of the same cohort showed that administering DLI based on lineage specific MC has a comparable GVHD risk profile to DLI administration based on MC in WB (33).

In a study of childhood ALL, IT was guided by MC and MRD (positive MRD and/or 1% MC) or by MRD only with CC (34). In this study, the 3-year EFS and 3-year incidence of relapse were similar in CC and MC patients with IT, whereas MC patients without IT experienced higher incidence of relapse. Notably, in this series, IT was not associated with an increased risk for acute GVHD. Similarly, another retrospective multicenter study including pediatric and adult patients with acute leukemia who received DLI after allogeneic HCT indicated that administration of DLI for cases with MC showed significantly prolonged OS compared to DLI administered for overt hematological relapse (96). However, GVHD and intervention-related death have been reported with pre-emptive IT (95).

Taken together, chimerism monitoring at day +30 has a clinical utility in predicting the EFS and relapse regardless of the sensitivity of testing method. Serial monitoring of chimerism using testing methods with high sensitivity (< 1%) has a clinical utility in guiding pre-emptive IT decisions. Considering the dynamic nature of chimerism state, relative changes in MC over time maybe more informative than absolute MC results at a given time point, however, more studies are needed to validate this observation.

Chimerism Analysis in Multiple Myeloma

In the setting of multiple myeloma, alloHCT may be indicated in cases of high-risk or refractory disease, harnessing a graft-versus-myeloma effect against malignant plasma cells (97–100). The role of chimerism analysis in these patients is not well studied. Kröger et al demonstrated delayed full DC of plasma cells, compared to T cells, and reported that serial plasma cell chimerism (CD138+ enriched samples) analysis by qPCR could be used in prediction of relapse (101). In contrast, a small retrospective study by Galimberti et al, found no correlation between DC as measured by STR analysis and overall- or progression-free-survival; but did identify an association between full DC and cGVHD (102). More recently, in a large (n=155) retrospective study of serial chimerism analysis by STR, loss of full DC was seen in only 36% of patients who experienced relapse or disease progression. A high proportion of patients who suffered relapse still had full DC as measured by PB or BM. Among these relapsed patients, almost one-third had extramedullary progression of the disease (103). A more recent study of post-alloHCT MM relapse had similar findings, although the chimerism methodology used was not described (104). These findings suggest that chimerism analysis outside of engraftment monitoring is of limited use post-alloHCT for MM. However, prospective studies, with consideration for how clinical interventions (DLI, immunosuppression tapering, post-transplant consolidation) could be informed by chimerism analysis results are needed to assess these retrospective data.

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Testing for HLA loss Relapse

HLA loss relapse occurs predominantly in haploidentical transplants.. Since the recipient mismatched HLA haplotype is a target for donor T cell mediated GVL occurring in patients who receive HLA haplo-identical transplants, loss of these HLA targets is a tumor escape mechanism to evade the GVL selective pressure. This loss of GVL activity of the donor T cells against the relapsed leukemic blasts compared to the original leukemic cells was demonstrated *in vitro* by mixed lymphocyte cultures(9, 105). The incidence of HLA loss relapse in haploidentical HCT was reported ranging from 14–33% (105–107).

Polymorphic markers within the HLA loci have been investigated as markers of post-HCT relapse, specifically the absence of a mismatched recipient HLA haplotype or HLA-loss relapse (8). In our survey, the majority of responses indicated the lack of testing for HLA-loss relapse. However, many responses expressed interest in the test when it becomes available at their institutions.

Importantly, this HLA loss has also been identified following HCT using other donor sources including well-matched unrelated donors, albeit this is uncommon in this setting (4, 107–109). Consistent with common practices of unrelated donor selection, a well-matched HCT donor that is 8/8 match at loci HLA-A, -B, -C and -DRB1 may harbor HLA mismatches in other HLA loci, commonly DPB1 and possibly DQB1 (110). In addition, few studies have investigated HLA loss in CBT, however, the frequency of this phenomenon remains unclear (107, 111). In terms of utilization, when samples are positive for MC by non-HLA markers, chimerism testing by HLA-markers can make the distinction between HLA loss relapse and the classical relapse, which could inform therapeutic decisions including whether to administer a DLI or select another donor for a re-transplant procedure. It has been suggested that in cases of HLA loss relapse DLI might not be effective. In addition, a favorable donor for a re-transplant needs to be mismatched for an HLA haplotype other than the recipient lost HLA haplotype to maintain a target for the GVL effect (112).

Limitations

The voluntary nature of participation in the survey and relatively low % of participation may have skewed the results. However, there was still reasonable representation from various parts of the world and practice types among members of CIBMTR. Furthermore, a significant proportion of the literature investigating chimerism was published more than 5 years ago and may not be reflective of evolving HCT clinical practices or recent advances in testing methods. In addition, the heterogeneity of reviewed literature spanning decades of evolving HCT clinical practices and testing techniques may have confounded inter-study comparisons. However, when pertinent, we highlighted the methodological differences across different studies. Finally, the majority of studies on failure of engraftment did not report on the presence of donor specific HLA antibodies (DSA) which is increasingly being recognized as a risk factor for primary failure of engraftment (113).

Concluding remarks and future directions

This review highlights the remarkable heterogeneity in chimerism analysis practices worldwide as well as findings and conclusions of published studies in this area. Although

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currently the most commonly used testing method is STR, recent studies support the utility of more sensitive methods such as NGS, especially for early detection of impending relapse. The method should be capable of detecting microchimerism, defined as MC <1% (10, 31). There is no conclusive evidence that testing chimerism in BM is more clinically actionable than chimerism testing in PB, particularly when using a testing method of high sensitivity. Periodic testing of chimerism is advantageous in monitoring dynamic changes in MC over time, especially in non-myeloablative HCT. In detecting trends, the accuracy of detecting changes in chimerism over time is more important than the sensitivity in determining chimerism level at a single time point. This raises the question as to the optimal cut-off for minimum increment of increase to predict relapse. There is no universally actionable cutoff for predicting relapse and there will always be a tradeoff (positive predictive value vs time to relapse) to use a high value with very stringent constraints will reduce the time-to-act.

More frequent testing may be warranted as part of diligent monitoring in patients with high risk of relapse such as AML. Testing lineage specific chimerism, particularly for T cells and possibly CD34+ is more sensitive in predicting impending relapse than testing whole blood. There is a suggestion of the utility of chimerism testing in other cell lineages, however, this utility may be related to specific clinical contexts rather than being universally applicable. Using chimerism studies to identify cases of HLA loss relapse is a more recent development in the field. Although not yet widely available, detection of HLA loss relapse provides relevant information pertinent to identifying patients in whom a particular donor mismatch is less likely to elicit a GVL effect. In order to have an informed decision on whom to monitor and when to test, studies determining the risk factors for HLA loss relapse are needed. In the absence of these studies, we recommend testing for HLA loss in post-haplo transplant patients with MC by non-HLA markers, where the highest frequency was reported compared to other graft sources. It may also guide the selection of more desirable donors for a potential second transplant. These guidelines are summarized in Table 3.

We submit that these guidelines are our interpretation of the sparse and heterogeneous literature on the subject and that is influenced by experiences and practices in our laboratories. We believe that it is prudent to reignite the work initiated by ASBMT and IBMTR over 20 years ago by designing another working group to develop formal recommendations for the clinical utilization of chimerism analysis based on evidence from published literature and expert opinions. This working group would also identify clear gaps of knowledge in this area and inform the design of studies to establish the most actionable attributes of chimerism analysis. These studies would determine which lineage(s) to test and at what time points. It’s even more critical to determine appropriate thresholds in terms of percent MC or kinetics over time to guide consideration of therapeutic intervention such as immunosuppression tapering or DLI administration. Ideally this working group would include representatives of clinical and technical stake holders including representatives of CIBMTR, National Marrow Donor Program (NMDP)/Be The Match, ASTCT, European Group for Blood and Marrow Transplantation (EBMT), International Society for Cell & Gene Therapy (ISCT), American Society for Histocompatibility and Immunogenetics (ASHI), European Federation for Immunogenetics (EFI) and College of American Pathologists (CAP).

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Supplementary Material

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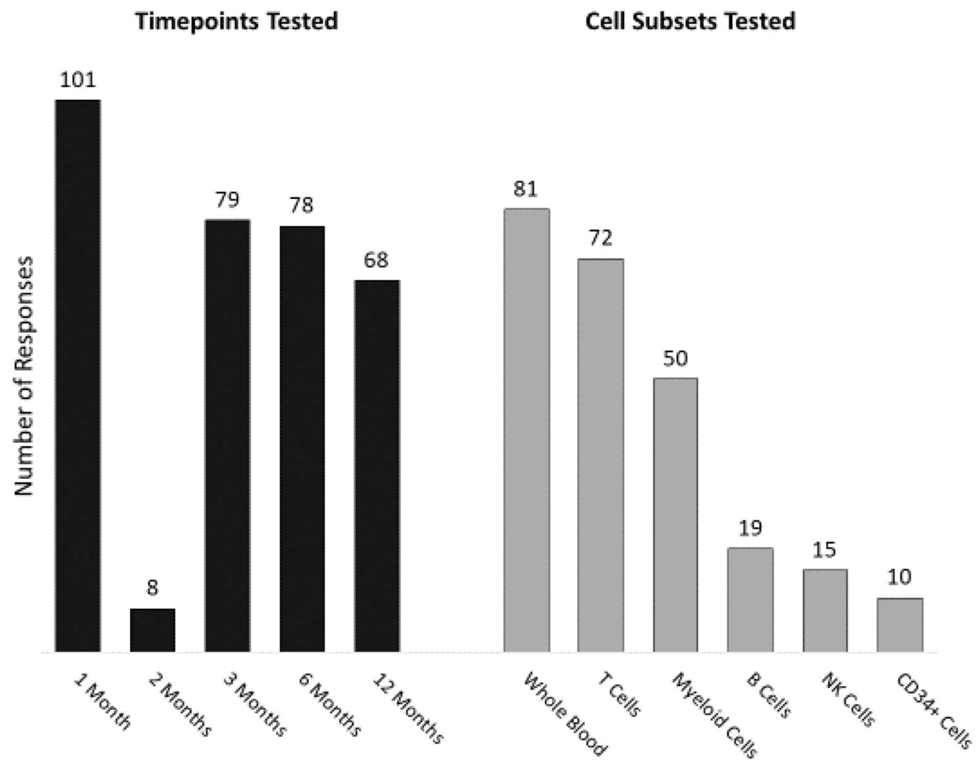

Figure 1: Time Points and Cell Subsets Tested by Chimerism Analysis, Survey Responses.
Left, Time points post-alloHCT in months. *Right*, Cell subsets tested post-alloHCT. Cell markers used to define subsets varied and included the following: T cell markers reported include: CD3, CD4, CD8; myeloid markers include: CD33, CD66b, CD15; B cell markers include: CD19, CD20; NK cell markers include: CD16, CD56.

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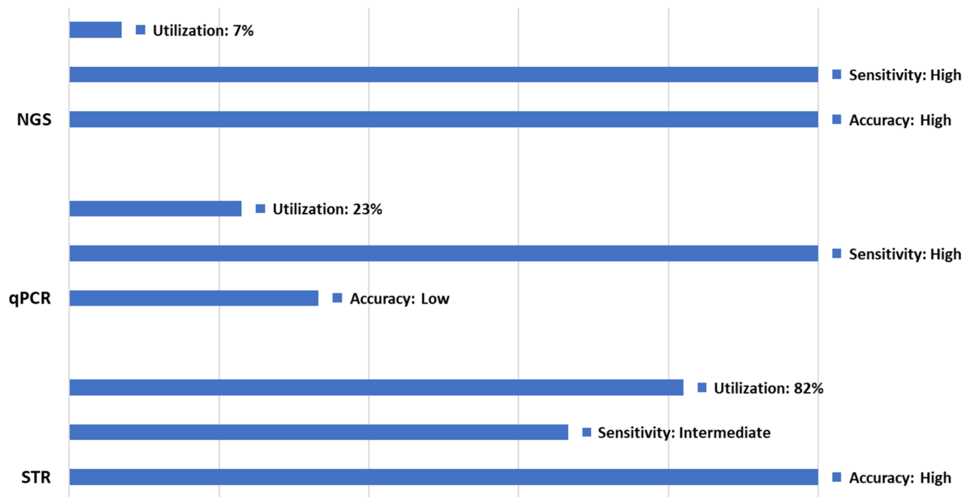

Figure 2: Accuracy, Sensitivity, and Current Utilization of Top 3 Survey-Reported Chimerism Analysis Methods.

Utilization is based on survey results; accuracy and sensitivity are based on published literature.

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Table 1:

Demographics of survey-respondent HCT programs.

Country of Practice	%	N
Europe & UK	19%	20
North America	48%	52
OTHER	33%	36
Patient Population	**%**	**N**
Adults	50%	54
Pediatrics	22%	24
Both	28%	30
Program Size	**%**	**N**
<=50	51%	55
51–100	36%	39
>100	13%	14
Types of Transplants Performed	**%**	**N**
HLA identical	100%	108
HLA haploidentical	99%	107
HLA matched unrelated donor	93%	100
HLA mismatched unrelated donor	75%	81
Single cord blood donor	52%	56
Double/multiple cord blood donors	44%	47
Chimerism Testing Laboratories	**%**	**N**
University Hospital/Academic Institute-based	79%	85
Other hospital-based	13%	14
Private/reference laboratory	6%	7
Government	5%	5

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Table 2:

Diagnostic value of host chimerism for the detection of relapse at different cut-off levels, based on a qPCR chimerism methodology.

	Sensitivity %	Specificity %	PPV %	NPV %	Median days between exceeding cut-off and relapse[a] (range)
Combination of C2 (%) and ICF					
C2 > 0.05 and ICF >1.010	85	80	21	99	45 (0–112)
C2 > 0.10 and ICF > 1.020	**68**	**89**	**28**	**98**	**20 (0-112)**
C2 > 0.50 and ICF > 1.040	32	97	39	96	0 (0–45)
Absolute host chimerism C2 at a cut-off of 0.10%					
t 14	79	72	17	98	38 (0–112)
t 21	82	75	24	98	17 (0–112)
t 28	85	82	28	98	17 (0–112)
t 35	87	83	42	98	17 (0–112)
t 42	93	80	50	98	0 (0–112)
t 49	91	85	54	98	0 (0–112)
Host chimerism increment factor ICF at a cut-off of 1.020					
t 14	84	79	23	98	45 (0–112)
t 21	82	81	29	98	17 (0–112)
t 28	85	88	59	99	17 (0–112)
t 35	84	90	54	98	14 (0–112)
t 42	89	91	69	97	0 (0–112)
t 49	91	93	72	98	0 (0–112)

[a]The median days between exceeding the respective cut-offs and disease relapse were applied to values followed by a steady increase in host chimerism until relapse

This table is modified from Sellmann *et al.* (93) and lists performance characteristics of C_2 , ICF combined at several different cutoffs. C_2 is the absolute value of host chimerism at subsequent time point, and ICF is an increment factor used to describe the relative change in host chimerism levels over time. Included are sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV). Also shown is the effect of the duration (*t*, days) of steadily increasing host chimerism on the performance characteristics of C_2 and ICF at the optimum cut-off of 0.10% and 1.020, respectively.

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Table 3:

Summary of Chimerism Testing Guidelines.

Indication	Relevance	High Sensitivity Method	Time Points	Testing for HLA Loss Relapse
Engraftment monitoring	Particularly in non-myeloablative transplants and those with increased risk of delayed or failed engraftment	Not necessary	+30 / +60 (for delayed engraftment)	Not relevant
Detection of Relapse	Particularly in diseases with high risk of relapse	Advantageous	When clinically suspected	Advantageous particularly in haplo-transplants