To the Editor:

Identification of immunoglobulin (IG) and T-cell receptor (TR) gene rearrangements in acute lymphoblastic leukemia (ALL) patients at initial presentation is crucial for monitoring of minimal residual disease (MRD) during subsequent follow-up and thereby for appropriate risk-group stratification. In a diagnostic setting, IG/TR gene rearrangements are generally identified using DNA-based PCR analysis, followed by classical Sanger sequencing or next generation sequencing (NGS) [1, 2]. Nowadays, whole transcriptome RNA sequencing (RNAseq) is frequently used to identify fusion genes and to assign patients into distinct molecular subgroups according to WHO 2016 classification, or for protocol-based clinical decisions [3]. Hence, it would be beneficial if RNAseq data could also be used for the identification of IG/TR gene rearrangements pertaining to the leukemic clone.

Recently, Yeoh and colleagues reported on the use of RNAseq data for identification of IG heavy chain (IGH) gene rearrangements, which was successful in approximately 90% of B-ALL patients [4]. Almost two-thirds of clonal IGH rearrangements were unproductive, whereas the vast majority (>98%) of background rearrangements were productive. Even though these data are promising, they are also incomplete since other IG/TR loci were not evaluated. Furthermore, these data underline that caution is warranted in the analysis of RNAseq data for IG/TR marker screening in ALL (and in other lymphoproliferative disorders requiring multiple RNA/DNA analyses): applying computational methods that only focus on productive rearrangements (e.g., like for most repertoire analyses) will clearly result in incomplete interpretation of IG/TR data for marker identification.

Within the EuroClonality-NGS Working Group and EuroMRD, we have developed, validated, and published assays for IG/TR DNA amplicon (‘DNAamp’ hereafter) and DNA capture-based analysis of relevant samples [1, 7, 8]. In line with these recent efforts, we decided to explore the possibilities and limitations of extracting data on IG/TR gene rearrangements from RNAseq data. Here we report on a complete but preliminary analysis of all IG/TR loci from RNAseq data, using DNAamp data as benchmark.

RNAseq data from 165 ALL patients at time of diagnosis were obtained using Illumina TruSeq Library Prep or Universal RNAseq kit (Nugen, Tecxan) and sequenced by Illumina Next-Seq or Novaseq (2 × 75 bp). DNAamp IG/TR
Table 1 IG/TR rearrangements detected by DNA amplicon-based methods (DNAamp) and by RNAseq.

| Rearrangements/case | DNAamp | RNAseq ("damaged") | RNAseq Productive | RNAseq MRD markers according to DNAamp benchmark |
|---------------------|--------|---------------------|-------------------|-----------------------------------------------|
|                     |        |                     |                   | DNAamp | RNAseq | RNAseq (%) |
| IGH complete VJ:Vh-(Db)-Jh | 2241   | 643 (215)           | 364               | 223    | 188    | 84.3        |
| IGH incomplete DJ:Db-Jh | 1407   | 21 (9)              | na                | 89     | 69     | 77.5        |
| IGK complete VJ:Vk-Ik | 762    | 436 (61)            | 323               | 63     | 23     | 36.5        |
| IGK other Vk-Kde | 285    | <1                  | na                | 57     | 2      | 3.5         |
| IGK other intron-Kde | 780    | <1                  | na                | 45     | 1      | 2.2         |
| IGL complete VJ:Vl-Jl | na     | 329 (53)            | 233               | na     |         |             |
| TRA complete VJ:Va-Ja | 11     | 62 (20)             | 25                | 0      |         |             |
| TRA + D complete VJ:Vd-Jd | 10     | <1                  | <1                | 0      |         |             |
| TRA complete VJ:Vd-(Db)-Ja | 133    | 2 (1)               | <1                | 33     | 14     | 42.4        |
| TRA + D incomplete DJ:Db-Ja | 71     | 37 (25)             | na                | 27     | 1      | 3.7         |
| TRA + D incomplete VD:Va-Dd3 | na     | <1                  | na                | na     |         |             |
| TRB complete VJ:Vb-(Db)-Jb | 1921   | 128 (34)            | 72                | 101    | 53     | 52.5        |
| TRB incomplete DJ:Db-Jb | 1555   | 13 (6)              | na                | 71     | 19     | 26.8        |
| TRB incomplete VD:Vb-Dh | na     | 1 (<1)              | na                | na     |         |             |
| TRB other DD:Db-Dh | na     | <1                  | na                | na     |         |             |
| TRD complete VJ:Vd-(Db)-Jd | 184    | 2 (<1)              | 1                 | 17     | 4      | 23.5        |
| TRD complete DJ:Db2-Jd | 139    | 1 (1)               | na                | 11     | 1      | 9.1         |
| TRD complete DJ:Db3-Jd | na     | <1                  | na                | na     |         |             |
| TRD incomplete VD:Vd-Dd3 | 188    | <1                  | na                | 82     | 1      | 1.2         |
| TRD other DD:Db2-Dd3 | 74     | 0                   | na                | 36     | 0      | 0           |
| TRD other DD:Db3-Dd2 | na     | <1                  | na                | na     |         |             |
| TRG complete VJ:Vg-Jg | 2163   | 12 (2)              | 3                 | 198    | 91     | 46          |

*Average number of rearrangements per case as detected by DNAamp and RNAseq. The RNAseq data refer to total number of rearrangements (with in between brackets the number of “damaged” sequences) and to the number of productive rearrangements. A total of 165 ALL patients were evaluated.

*Number of potential MRD markers identified by DNAamp (as per abundance thresholds), and number and percentage of those markers identified by RNAseq at any abundance. A total of 165 ALL patients were evaluated.

*na: not analyzed/not applicable.
each locus (Fig. 1A): the DNAamp data provide an average of 1703 rearrangements per case per locus (range 11–3648) compared to 211 (range 3–664) for RNAseq. When “damaged” rearrangements were ignored, RNAseq numbers fell to 157 (range 2–440). The most noticeable difference in relative incidence is the remarkable under-representation of TRB, TRD and TRG gene rearrangements in the RNAseq data. Furthermore, of the rearrangements identified by RNAseq, the vast majority is complete (96%) and the absolute majority is productive (>60%; Fig. 1B). In contrast, by DNAamp the complete rearrangements are relatively less frequent (<60%) and nonproductive rearrangements are found in about 40% of patients.

The lower number of total IG/TR rearrangements detected by RNAseq as compared to DNAamp also limited the identification of potential MRD markers. Figure 1C and Table 1 show the absolute number of potential MRD markers above the aforementioned abundance thresholds in the DNAamp data, and results from using DNAamp as benchmark for the sensitivity of RNAseq to identify MRD markers. At present, the non-trivial question of appropriate thresholds for marker screening in RNAseq has not been answered. Therefore, we decided to search for the respective MRD marker obtained by the DNAamp approach in the RNAseq data at any abundance. This probably means that our results are overestimating the ability of RNAseq to recover DNAamp markers. Overall, less than half of the DNAamp markers were found in the RNAseq data (467/1053; 44%). Best concordance was observed for IGH (complete and incomplete) markers (82% of DNAamp markers also identified by RNAseq); other complete markers (IGK, TRB, TRA+D, TRD and TRG) were partially detected by RNAseq (24–52%), while most incomplete markers and virtually all IGK-Kde markers were only limitedly identified by RNAseq (<10%). Evaluation of the individual ALL cases showed that in eight out of 165 cases (5%) none of the DNAamp markers were found in the RNAseq results (again, at any abundance), while identical marker profiles were seen in only 19/165 cases (12%). In the remaining 138 patients, at least some of the potential markers were missed. When the same abundance criteria were used for both DNAamp and RNAseq, by RNAseq, 38 cases (23%) had one potential marker, 50 cases (30%) two, and 37 cases (22%) three (and seven cases had none); for DNAamp these numbers were six (4%), three (2%), and 14 (8%), respectively. The remaining cases (i.e., 33 (20%) in RNAseq, 142 (86%) in DNAamp) had four or more potential markers available. Thus, at least 2 IG/TR markers (required in most current clinical protocols) were identified by DNAamp in 159/165 (96%) cases and by RNAseq in 120/165 cases (73%).

Our preliminary data thus show that IG/TR rearrangements detected by DNA amplicon-based methods are clearly distinct from RNAseq-identified rearrangements. Obviously, this is to a large extent explained by the underlying (immuno)biology of IG/TR gene rearrangements, which will
mainly be transcribed if complete and productive, thus allowing production of a functional IG/TR chain. In contrast, non-transcribed unproductive and incomplete rearrangements are hardly or not detectable by RNAseq. Of importance, about 70% of IGH rearrangements in ALL patients are unproductive, whereas non-leukemic rearrangements generally are productive [4, 6]. While it is appropriate to filter out out-of-frame immune rearrangements in analysis of functional repertoires, this is certainly not appropriate for ALL IG/TR marker analysis [5]. In our analysis, we did not yet take full transcription levels into account (i.e., we counted in de-duplicated reads, or unique fragments, as in DNA capture analyses), but it will be interesting to evaluate whether for example TR rearrangements in reactive T-cell clones show differences in transcript levels compared to cross-lineage TR rearrangements in B-cell precursor type ALL. From a technical perspective, it should be noted that we applied 2 × 75 bp sequencing and it may be expected that IG/TR rearrangement detection will be improved if longer reads are used. From a clinical perspective, RNAseq data may be used for MRD marker identification in those cases where DNAamp methods are unsuccessful or unavailable, if appropriate bioinformatic strategies are used.

Of note, IG/TR rearrangements may also be derived from whole-exome sequencing (WES) or whole-genome sequencing (WGS) datasets that, in contrast to RNAseq data, do not depend on the transcriptional level of rearrangements. This creates a clear advantage as was recently showcased in work introducing IgCaller for WGS-derived IGH data [11].

We therefore plan to continue and expand on the evaluation of not only RNAseq, but also WES and WGS data, mainly in ALL but also in NHL, in comparison to results obtained from a large set of DNAamp and targeted DNA capture data from the EuroClonality-NGS/EuroMRD groups. Challenges will include compiling appropriate rules and thresholds for marker identification, preparing specific protocols and suggesting limits for the safe use for each technology, for example and especially for MRD monitoring.

**Acknowledgements** GC was supported by a grant from the Italian Association for Cancer Research (AIRC), grant IG2015-17593. BS was supported by a grant from the Dutch Cancer Society (2017/11137).

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### Compliance with ethical standards

**Conflict of interest** The EuroClonality-NGS Working Group is an independent scientific subdivision of EuroClonality that aims to innovation, standardization and education in the field of diagnostic clonality analysis. The revenues of the previously obtained patent (PCT/NL2003/000690), which is collectively owned by the EuroClonality Foundation and licensed to InVivoScribe, are exclusively used for EuroClonality activities, such as for covering costs of the Working Group meetings, collective WorkPackages and the EuroClonality Educational Workshops. The EuroClonality consortium operates under an umbrella of ESLHO, which is an official EHA Scientific Working Group. VHvdV: contract research for Pfizer and Janssen, Service Level Agreements with BD Biosciences and Agilent. MB reports personal fees from Incyte (advisory board), financial support for reference diagnostics from Affimed, Amgen and Regeneron, grants and personal fees from Amgen (advisory board, speakers bureau, travel support), personal fees from Janssen (speakers bureau), personal fees from Molecular Health (advisory board), all outside the submitted work. AWL: contract research for Roche-Genentech, research support from Gilead, advisory board for AbbVie, speaker for Gilead, Janssen. The other authors declare no conflict of interest.

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