GENOMICS OF LYMPHOPROLIFERATIVE DISEASE

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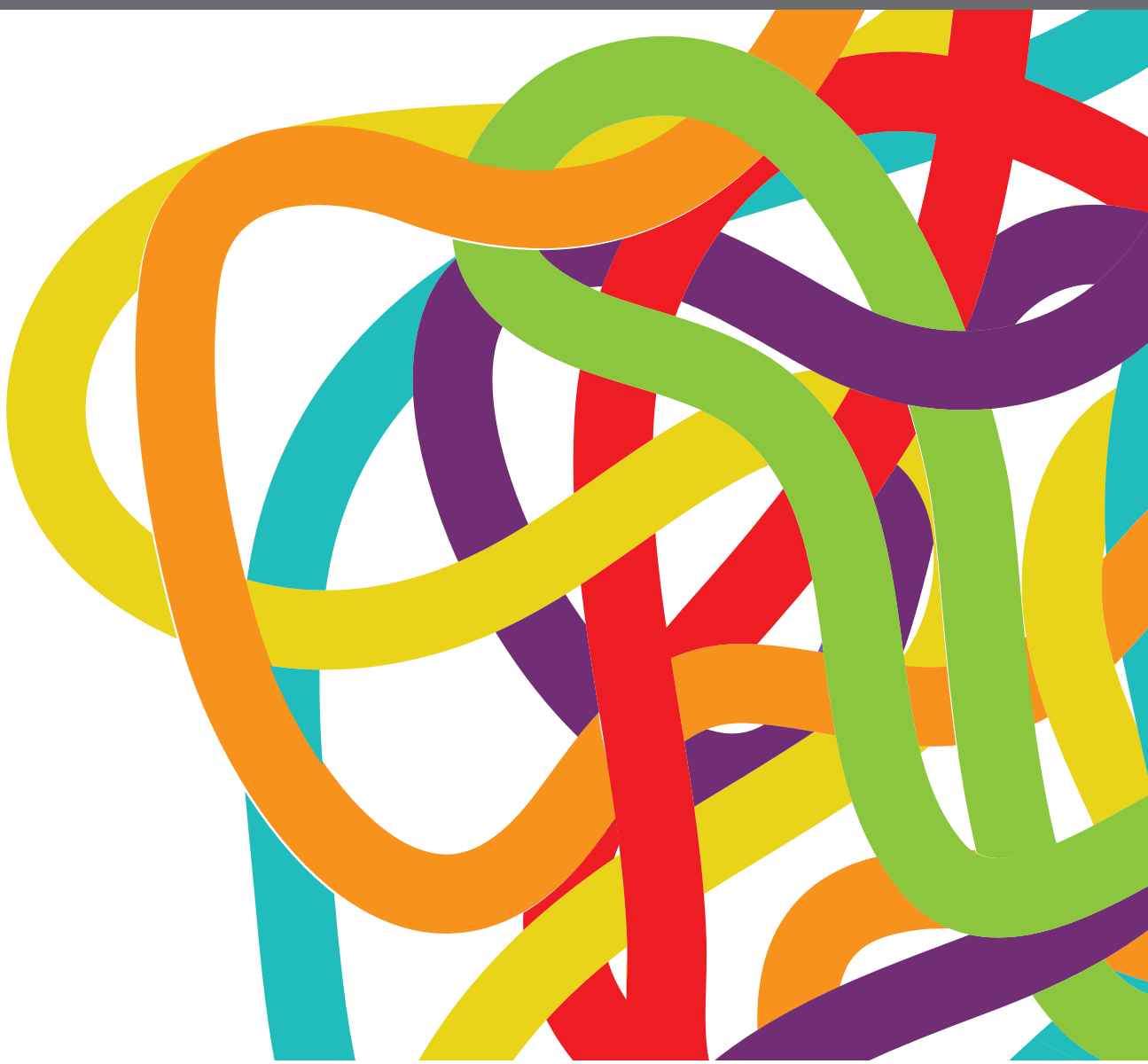

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GENOMICS OF LYMPHOPROLIFERATIVE DISEASE

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EDITORIAL
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Editorial: Genomics of Lymphoproliferative Disease

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Keywords: lymphoma, leukemia, multiple myeloma, lymphoproliferative disease, transcriptome analysis, gene mutation analysis, integrative "omics"

Editorial on the Research Topic

Genomics of Lymphoproliferative Disease

In the last years, next generation sequencing (NGS) technology has progressively revolutionized clinical and research approaches either to solid or hematological cancers. Taking advantage of an unprecedented resolution, scientists are currently able to comprehensively characterize several genomic and transcriptomic aspects of the cancer cell with both diagnostic and prognostic relevance. Recent studies have proposed different genomic classifiers to sharply stratify distinct tumor entities and define novel genetic drivers of cancer progression and resistance to current therapies. Our appreciation of the transcriptome complexity and the role of small, long and circular RNAs in disease mechanisms is continuously increasing, expanding the repertoire of potentially druggable targets. Indeed, several relevant and fascinating challenges are about to be addressed in the coming years: identifying the functional and biological significance of the driving genetic events; translating fundamental science into daily diagnostic clinical practice; finding novel prognostic markers and therapeutic targets to ultimately implement precise, effective and cost-saving therapies for patients with leukemia or lymphoma tumors.

In the Research Topic "Genomics of Lymphoproliferative Disease", we collect the contributions to the field from outstanding Researchers investigating lymphoid malignancies with "-*omics*" tools.

Diffuse large B-cell lymphoma (DLBCL) represents the most common histotype of lymphoma and to date remains still incurable for a large fraction of patients. A deeper characterization of DLBCL is the basis of a better knowledge of the disease for patients' care. In their Review, Cascione et al. highlighted the new molecular classifications of diffuse large B cell lymphomas, with emphasis on copy number variation. The Authors analyze two specific aspects, namely the transformation from indolent lymphoma and diffuse large B cell lymphoma in immunodeficient patients, providing the scientific community with a useful and essential overview of the major molecular classifications described so far. Opinto et al. explored the opportunities offered for the functional characterization of the tumor microenvironment in lymphoma pathogenesis, highlighting the translational value of "-omics" investigations in DLBCL and examining how the interplay between mesenchymal and hematopoietic counterparts in secondary lymphoid organs governs tumor evolution, with peculiarities of different disease subtypes and relevant prognostic implications.

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A perspective on the role in Anaplastic Large Cell Lymphoma (ALCL) of small RNA (sRNA) cargos transported by circulating extracellular vesicles was provided by Lovisa et al. There is high interest in studying exosomes of cancer patients both to develop non-invasive liquid biopsy tests for risk stratification and to elucidate their possible involvement in disease mechanisms. RNA-seq profiling of the content of circulating exosomes of ALCL pediatric patients and healthy controls disclosed that non-miRNA derived sRNAs constitute a prominent fraction loaded in exosomes and identified those sRNAs significantly more abundant in exosomes of ALCL patients than in controls. Further investigation identified *RNY4* massive loading into exosomes of ALCL patients, particularly those with advanced and aggressive disease. In ALCL, emerging data indicate that circulating YRNA can reflect the biology of the tumor or the patient's immune system reaction and prompt a further study of *RNY4* involvement in ALCL tumor microenvironment and disease aggressiveness. A novel oncogenic fusion gene *MEIS1–FOXO1* was described in pediatric B-cell acute lymphoblastic leukemia (ALL), showing that a reduced *FOXO1* expression is associated with unfavorable chemoresistant ALL subtype at high risk of relapse (Zheng et al.). Fattizzo et al. provided a focus on the physiopathology of T-cell acute lymphoblastic leukemia (T-ALL). Despite recent significant progress, the biology and the molecular drivers responsible for T-ALL aggressive clinical courses are not fully deciphered. Considering the large availability of new therapies (e.g. small molecules, CAR-T and monoclonal antibody), the Authors discussed the importance of molecular aspects with clinical, prognostic, and therapeutic significance.

Multiple myeloma (MM) was the subject of several articles, particularly in relation to the opportunities to improve disease monitoring offered by genomics. The critical importance of minimal residual disease (MRD) evaluation in MM was examined (Oliva et al.). The integration of MRD assessed by next-generation flow cytometry or next-generation sequencing is emerging as a key endpoint for the multiple myeloma community. In this review, authors addressed many questions regarding the future clinical use of MRD. The excellent review of Bolli et al. tackled the feasibility of NGS-based approaches for detailed genomic and transcriptomics characterization of MM in individual patients, to achieve an effective personalized treatment and management. The benefits and pitfalls of NGS-based diagnostics are analyzed in the paper, highlighting crucial aspects that must be considered for diffuse implementation. In MM, routine clinical markers can capture a few main high-risk features but are "blind to" most genomic lesions, that are particularly numerous and highly heterogeneous in this malignancy. Moreover, available evidence of clonal evolution in MM, with changes in sub-clonal structure and enrichment of high-risk features is pushing the clinics to the use of NGS-based methods to collect molecular data more informative also of disease evolution and resistance. Escape of MM to currently available therapies is the focus of Manni et al., who focus on MM cell ability to adapt to different chronic stress factors and discuss actionable molecules and pathways related to cellular fitness and stress resistance that can be targeted to treat the disease. High-throughput studies, such as structural genomics and transcriptomics, indicated potential vulnerable targets of MM biology. Additionally, major evidence obtained by RNAi, CRISPR/CAS9 and high throughput drug screenings highlighted novel putative regulators of MM cell survival and resistance to stress that could be therapeutically targeted.

Other original articles provide interesting updates also on less frequent entities, highlighting the most recent discoveries and discussing largely unknown aspects of their pathogenesis. Fattizzo and Barcellini summarize what we know about the connection between chronic lymphocytic leukemia and autoimmune complication. Ferla et al. explored the biology of post-transplant lymphoproliferative disorders (PTLD), a rare complication whose genomic background remains largely unknown. Finally, Teramo et al. provide a detailed summary of the genetic lesions involved in Large Granular Lymphocyte Leukemia pathogenesis.

AUTHOR CONTRIBUTIONS

FM, LA and SB equally contributed to draft and revise the editorial. The missing Topic editors have read and approved the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Autoimmune Cytopenias in Chronic Lymphocytic Leukemia: Focus on Molecular Aspects

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Autoimmune cytopenias, particularly autoimmune hemolytic anemia (AIHA) and immune thrombocytopenia (ITP), complicate up to 25% of chronic lymphocytic leukemia (CLL) cases. Their occurrence correlates with a more aggressive disease with unmutated VHIG status and unfavorable cytogenetics (17p and 11q deletions). CLL lymphocytes are thought to be responsible of a number of pathogenic mechanisms, including aberrant antigen presentation and cytokine production. Moreover, pathogenic B-cell lymphocytes may induce T-cell subsets imbalance that favors the emergence of autoreactive B-cells producing anti-red blood cells and anti-platelets autoantibodies. In the last 15 years, molecular insights into the pathogenesis of both primary and secondary AIHA/ITP has shown that autoreactive B-cells often display stereotyped B-cell receptor and that the autoantibodies themselves have restricted phenotypes. Moreover, a skewed T-cell repertoire and clonal T cells (mainly CD8+) may be present. In addition, an imbalance of T regulatory-/T helper 17-cells ratio has been involved in AIHA and ITP development, and correlates with various cytokine genes polymorphisms. Finally, altered miRNA and lnRNA profiles have been found in autoimmune cytopenias and seem to correlate with disease phase. Genomic studies are limited in these forms, except for recurrent mutations of KMT2D and CARD11 in cold agglutinin disease, which is considered a clonal B-cell lymphoproliferative disorder resulting in AIHA. In this manuscript, we review the most recent literature on AIHA and ITP secondary to CLL, focusing on available molecular evidences of pathogenic, clinical, and prognostic relevance.

Keywords: autoimmune hemolytic anemia, immune thrombocytopenia, chronic lymphocytic leukemia, Evans' syndrome, molecular

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INTRODUCTION

The impact of autoimmune cytopenias (AIC) complicating chronic lymphocytic leukemia (CLL), particularly autoimmune hemolytic anemia (AIHA) and immune thrombocytopenia (ITP) is variable, ranging from mild asymptomatic cytopenias case without indication to CLL treatment, to severe transfusion dependent patients with abrupt onset and CLL progression. Each patient needs to be carefully evaluated, since the different pictures require a specific approach. Given this heterogeneity, the variability of response to immune-suppression, and the possible association/development of clonal diseases (lymphoproliferation or myelodysplasia), the genomic landscape of AIC is of particular interest.

In this manuscript, we will review the most recent literature on AIHA and ITP secondary to CLL with a brief summary of their clinical management. In particular we will focus on available molecular evidences of pathogenic, clinical, and prognostic relevance.

EPIDEMIOLOGY AND PATHOGENESIS

AIC may complicate CLL course at any time, from diagnosis to disease progression (**Figure 1**) (1). AIHA are the most frequent form (7–10% of cases), followed by ITP (1–5%), and rarer entities such as pure red cell aplasia (PRCA, <1%) and autoimmune granulocytopenia (AIG 0.17%). From a pathogenic point of view, CLL associated AIC are mediated by a complex orchestration of humoral, cellular, and innate immunity: (1) IgG auto-antibodies coat erythrocytes, platelets, and neutrophils with consequent antibody-dependent cellular cytotoxicity and complement-mediated destruction in the reticuloendothelial system (spleen and liver) or in the blood stream. (2) Anti-erythroblast and megakaryocyte autoantibodies can impair bone marrow compensatory response. (3) Autoreactive T-cells produce inflammatory cytokines and further inhibit myelopoiesis. (4) Natural killer cells have been shown to destroy erythroblasts from CLL patients *in vitro*, confirming a role for innate immunity.

As regards autoantibodies, they are polyclonal high-affinity IgG produced by non-malignant self-reactive B-cells in 90% of cases. CLL cells may also produce autoantibodies (mainly IgM) in <10% of cases (2–5), and have been shown to secrete soluble factors inducing a dysregulation of bone marrow microenvironment (6, 7). Further pathogenic mechanisms, are the direct antigen presentation by CLL cells that may induce self-reactive T helper cells, and the production of non-functional T regulatory cells (T-regs) (8–10). The latter become unable to eliminate non-neoplastic autoreactive T- and B-cells leading to autoimmune phenomena (11–14). In addition, an increased incidence of autoimmune cytopenias in CLL is associated to an imbalance in the ratio between Th17 cells and T-regs (15). Finally, CLL patients developing autoimmune phenomena displayed a reduction of Toll-like receptors (TLR)-4, an important player of the innate immunity, together with a lower expression of TLR2, and an increase of TLR7, TLR9, and TLR10 (16–18).

Influence of CLL Therapy on the Development of AIC

The influence of CLL therapy on the development of AIC deserves special consideration: single-agent purine analogs (i.e., fludarabine) may induce CLL-AIHA (19, 20) possibly worsening the imbalance between Th17 and T-regs (21). FC and FCR combination schemes (fludarabine, cyclophosphamide, and rituximab) in the CLL8 trial (22) showed very low incidence (<1%) of hemolytic anemia, as did bendamustine rituximab (BR) association (even if anecdotic PRCA cases have been described) (23). Alemtuzumab led to treatment-emergent ITP in 9% of CLL cases (24), again possibly due to T-cell dysregulation. Concerning small molecules, the most interesting data are available for Bruton's tyrosine kinase inhibitor ibrutinib: new-onset AIC was rarely reported in the largest studies performed so far (25–27). Moreover, AIC resolution occurred in about a half of CLL-AIC patients ($N = 13$) (26) and most CLL-AIC cases were able to discontinue AIC-therapy after a median of 4.7 months ($N = 301$ of whom 7% with ongoing AIC therapy) (27). Similar data were reported in a more recent

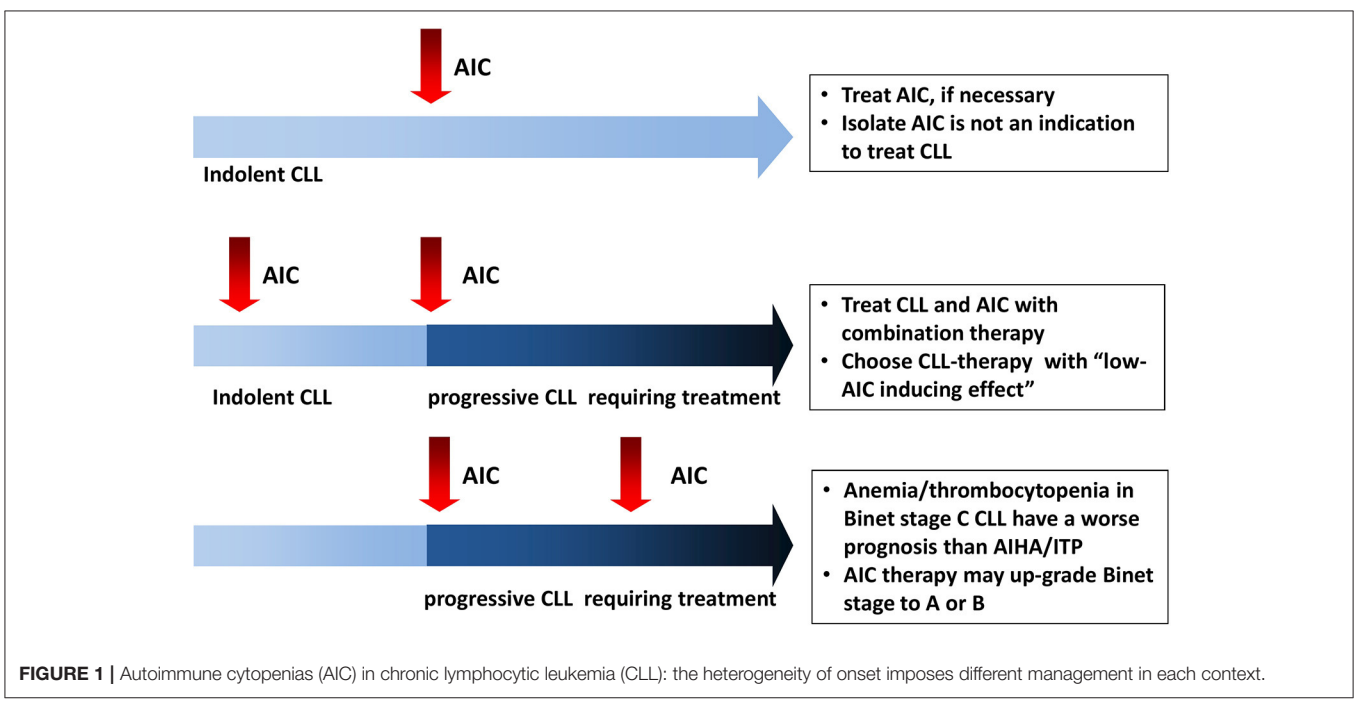

FIGURE 1 | Autoimmune cytopenias (AIC) in chronic lymphocytic leukemia (CLL): the heterogeneity of onset imposes different management in each context.

study of 193 patients: 67% of 29 cases with AIC pre-ibrutinib could discontinue/taper AIC treatment and new-onset AIC occurred in 6% (all with unmutated IGHV) (28). Recent evidences suggest an inhibitory role of ibrutinib on autoreactive T cells, through interleukin-2-inducible kinase (ITK)suppression, leading the way for its use in T-cell mediated autoimmune conditions (i.e., graft vs. host disease) (29). Regarding other small molecules, limited data are available for idelalisib (that targets phosphoinositide 3-kinase), and venetoclax (a BCL-2 antagonist), although the presence of autoimmune phenomena was an exclusion criteria in various trials. Concerning venetoclax, it has been reported to be associated to the occurrence, although rarely, of AIHA in large CLL registrative trials (30). Interestingly, increased incidence of autoimmune complications (hepatitis, colitis, and pneumonitis) has been reported for idelalisib (31, 32).

MANAGEMENT OF AUTOIMMUNE HEMOLYTIC ANEMIA SECONDARY TO CLL

Diagnosis

Management of AIHA in CLL requires the evaluation and exclusion of the other possible causes of anemia, including bone marrow infiltration/failure, bleeding, vitamin or iron deficiencies, and renal disease. As previously suggested, a diagnosis of AIHA can be established in the presence of Hb < 11 g/dL, no chemotherapy in the previous month, variable alteration of hemolytic markers (increased unconjugated bilirubin, elevated lactate dehydrogenase, consumption of haptoglobin, increased absolute reticulocyte counts), and the positivity of the direct antiglobulin test (DAT) (1, 33). The latter allow to distinguish warm (wAIHA: DAT positive for IgG or IgG+C3d at low titer and negative autoagglutination at 20°C) from cold (cAIHA) cases (DAT positive for C3d and positive autoagglutination at 20°C). Of note, CLL itself may be a confounder in the differential diagnosis, since LDH may be elevated during disease progression, haptoglobin increased due to chronic/acute inflammation, and reticulocytosis may be absent or inadequate due to bone marrow infiltration or suppression by cytokine storm and/or anti-erythroblasts antibodies (1). The latter, demonstrated in a proportion of CLL cases through the mitogen-stimulated DAT, were associated to increased IL-4 and IFN-γ production, and may contribute to ineffective erythropoiesis (34). Furthermore, DAT positivity does not necessarily mean AIHA and in a longitudinal study of DAT+CLL cases only one third developed clinically overt hemolysis (35). Conversely, DAT negative AIHA cases may also be present (36), possibly due to the low-affinity or to the very small number of autoantibodies. In this context, the use of more sensitive techniques (microcolumn and solid-phase tests, or mitogen-stimulated DAT) may be useful (34). Finally, Bone marrow biopsy is usually necessary to document CLL infiltration and to rule out other causes (including bone marrow failure).

Treatment

As regards therapy (**Table 1**), the acuteness of onset, the severity of the anemia and the degree of hemolysis should be considered, together with patient' symptoms, age and comorbidities. Blood transfusions are usually indicated if Hb < 6 g/dL or higher in elderly comorbid patients. Over-transfusion should be avoided since it carries high risk of allo-immunization. In CLL-cases, given underlying bone marrow impairment and inadequate reticulocytosis, transfusion requirement may be higher than

TABLE 1 | Specific therapies and relative outcomes for warm and cold autoimmune hemolytic anemia and immune thrombocytopenia secondary to chronic lymphocytic leukemia (CLL).

Treatment	Line	Overall response rate %	References
WARM AUTOIMMUNE HEMOLYTIC ANEMIA wAIHA			
Prednisone 1 mg/kg/day for 3–4 weeks	1st	84–90	(1, 37)
Dexamethasone 40 mg/day for 4 days, 2–6 cycles every 2–4 weeks	1st	100	
Rituximab 375 mg sqm weekly × 4	2nd or >	72–80	(38, 39)
Cyclosporine 3–5 mg/Kg day	3nd or >	56	(40)
Alemtuzumab 30 mg × 3/week × 4–12 weeks	3nd or >	100	(41, 42)
Splenectomy	3nd or >	69–78	(43)
COLD AUTOIMMUNE HEMOLYTIC ANEMIA cAIHA			
Rituximab 375 mg/sqm weekly × 4	1st	50–70	(1, 39, 44)
Rituximab+Bendamustine 90 mg/sqm	2nd or >	71–80	(45, 46)
Rituximab+Fludarabine 40 mg/sqm	2nd or >	76	(47)
IMMUNE THROMBOCYTOPENIA ITP			
Prednisone 1 mg/kg/day for 3–4 weeks	1st	90	(37)
Dexamethasone 40 mg/day for 4 days, 2–6 cycles every 2–4 weeks	1st	90	
Rituximab 375 mg sqm weekly × 4	2nd or >	78	(48–50)
TPO analog Romiplostim 1–10 mcg/Kg week Eltrombopag 50–150 mg day	3rd or >	80	(51–53)
Alemtuzumab 30 mg × 3 week × 4–12 weeks	3rd or >	100	(42)
Cyclosporine 3–5 mg/Kg day	3rd or >	62	(40)
Splenectomy	3rd or >	61	(43)
Other rituximab associations reported for warm and cold AIHA, and ITP Rituximab+cyclophosphamide and dexamethasone (RCD)	2nd or >	89	(54, 55)
Rituximab+cyclophosphamide, vincristine, and prednisone (R-CVP)	2nd or >	95	(56, 57)

Current guidelines suggest CLL-directed therapy in relapsed/refractory cases.

in primary cases. Moreover, the evaluation of endogenous erythropoietin (to be performed before repeated transfusions that may confound the picture) could suggest the use of recombinant erythropoietin. For warm AIHA, steroid therapy is considered the first line (usually prednisone at 1 mg/kg day for 3–4 weeks, followed by a slow tapering in a total of 6 months). Methylprednisolone boli (2–10 mg/Kg day for 3 days) may be considered, with or without intravenous immunoglobulins (0.4 g/kg for 5 days or 1 g/kg for 2 days), in patients with acute hemolysis and slow response to steroid therapy (1, 37). The fewer patients with cAIHA may have a milder clinical presentation with Hb levels >9 g/dL and cold agglutinin associated symptoms (acrocyanosis, itch, urticarial, etc.) and may require a watchful waiting approach. Treatment should be reserved for transfusion-dependent cases, active hemolysis (even if increase of LDH is difficult to judge in CLL), and invalidating cAIHA symptoms. Corticosteroids are usually effective only at high doses, and are a useful tool only in the acute setting. Prompt rituximab treatment should be considered, together with a quick steroid tapering after Hb stabilization. Rituximab is currently considered the first therapy line in cAIHA at standard dose of 375 mg/sm weekly for 4 weeks, with an overall response in up to 70–100% of patients (1, 39, 44). Considering patients refractory to first-line treatment (both wAIHA and cAIHA), current guidelines advice the introduction of a CLL directed therapy. The choice between chemoimmunotherapy and small molecules should be made according to current guidelines (patient age/comorbidities and CLL molecular characteristics) and considering potentially hemolytic side effects (avoid fludarabine single agent). As regards published studies specifically addressing refractory CLL-AIHA, rituximab in various combinations was able to induce high (>80%) and durable response rates: 89% ($N = 8$) with cyclophosphamide and dexamethasone (RCD) (54, 55), 95% ($N = 20$) with cyclophosphamide, vincristine, and prednisone (R-CVP) (56), and 80% with bendamustine ($N = 26$), with a median relapse free survival of 28 months (45, 46). Good results have also been reported in association with oral fludarabine, even if mainly in primary cAIHA cases (47). The only exception to this aggressive approach regards steroid-refractory wAIHA with no signs of CLL progression. In this setting, a possible strategy is to administer rituximab single agent with a reported efficacy in 72% of cases, of whom 40% sustained responses at 17 months (38, 39). Alemtuzumab has been abandoned because of serious infectious and autoimmune complications, as also happened for splenectomy (41–43). Cytotoxic immunesuppressors showed heterogeneous and weak efficacy in primary AIHA and are usually not administered in CLL secondary cases (40, 58). New generation monoclonal antibodies, such as of atumumab and obinutuzumab, may also be useful in secondary AIHA (59). As cited above, ibrutinib seems to be safe in patients with CLL-AIHA and progressive disease, and a phase II trial of ibrutinib combined to rituximab is ongoing in CLL-wAIHA [NCT03827603]. Regarding venetoclax, case reports of successful treatment have been published (60, 61).

MANAGEMENT OF IMMUNE THROMBOCYTOPENIA SECONDARY TO CLL

Diagnosis

The same diagnostic caveats mentioned for CLL-AIHA have to be considered in the thrombocytopenic patient. ITP should be suspected in a CLL patient with $<100 \times 10^9$/L platelets, with no chemotherapy in the previous month; moreover signs of CLL progression should be excluded (progressive splenomegaly, concomitant anemia, significant bone marrow CLL infiltrate, evidence of bone marrow failure/dysplasia). Other secondary causes (infections, drug-induced thrombocytopenia, thrombotic microangiopathies, and heparin-induced thrombocytopenia) should also be ruled out. Antiplatelet antibodies are of little aid due to the low sensitivity and specificity of the test, and usually not performed (1).

Treatment

ITP should be treated only in case of severe thrombocytopenia (Plt $< 30 \times 10^9$/L) or bleeding. First-line therapy with steroids (prednisone at 1 mg/kg day for 1 month, followed by a slow tapering, or dexamethasone 40 mg/day × 4 days 1–3 cycles) is the standard approach, with about 50% responders. Intravenous immunoglobulin can be added in case of bleeding or slow response to steroids, again with 50% response rate [(27)]. Platelet transfusion may be required in case of life-threatening hemorrhage. Similarly to CLL-AIHA, steroid refractory cases would deserve CLL-directed therapy evaluation. Rituximab monotherapy was shown effective in 86% of CLL-ITP cases (57% complete response) (48), with 21 months response duration (49, 50). Rituximab combined to cyclophosphamide and dexamethasone or to cyclophosphamide, vincristine and prednisone had a high rate of durable responses in published experiences (55, 57). Splenectomy is usually discouraged given the increased infectious risk, older age and comorbidities of CLL patients. Finally, thrombopoietin mimetics (romiplostin and eltrombopag), indicated in refractory primary ITP, have shown high (up to 80%) and durable responses in patients with CLL-ITP (51–53, 62).

MOLECULAR ASPECTS IN PRIMARY AND SECONDARY AIHA

Table 2 shows available studies addressing molecular aspects of warm and cold AIHA, both primary and secondary to lymphoproliferative disorders.

Studies on Immunoglobulin Genes

Since the autoantibody is the major pathogenic player, the larger and older experiments focused on the configuration of the genes of the variable region of the immunoglobulin heavy chains (IGHV) encoding AIHA autoantibodies and demonstrated that some rearrangements are preferentially involved. Almost all patients with cAIHA displayed monoclonal antibodies encoded

TABLE 2 | Molecular findings in primary and secondary autoimmune hemolytic anemia (AIHA) and Evans' syndrome.

Disease	Gene/Pathway	No. of patients	Technique	Impact and significance		References
PRIMARY AIHA						
Cold AIHA	IGHV4-21	2	Nucleotide sequence analysis	Pathogenic	VH4-21 gene segment is responsible for the major cross-reactive idiotype	(63)
Cold AIHA	IGHV region	–	Nucleotide sequence analysis	Pathogenic	Specific IGVH regions are related to anti- i and I red blood cell antigens autoantibodies	(64)
Cold AIHA	IGHV4-34	–	PCR	Pathogenic	Anti-RBC antibodies are clonally restricted	(65)
Cold AIHA	IGHV3-23	–	Selection of phage-antibody library on human red cells	Pathogenic	//	(66)
Cold AIHA	+3 and +12	–	Chromosome analysis	Pathogenic	Autoreactive B-cells are clonal	(67, 68)
AIHA	TNF-α, LT-α, IL-10, IL-12, CTLA-4	17	PCR and specific restriction enzyme digestion	Pathogenic/therapeutic	AIHA show higher frequency of LT-α (+252) AG phenotype	(69)
Cold AIHA	IGKV3-20 and IGKV3-15	27	IGH and IG light chain gene sequencing	Pathogenic/therapeutic	IGHV and IGKV correlate with cold agglutinin disease onset and activity	(70)
AIHA	TCRG and TCRB	33	DNA sequencing	Pathogenic/therapeutic	Pathogenic T-cells are clonally restricted in AIHA	(71)
Cold AIHA	KMT2D and CARD11	16	Exome sequencing, targeted sequencing, Sanger sequencing	Pathogenic/therapeutic	Autoreactive B-cells display somatic mutations favoring proliferation	(72)
SECONDARY AIHA						
AIHA in CLL	IGVH51p1	12	PCR	Pathogenic	CLL patients expressing IGVH51p1 are more prone to AIHA	(73, 74)
AIHA in CLL	IGHV1-69, IGHV3-11, IGHV4-59, HCDR3	319	RT-PCR	Pathogenic/prognostic	Sterotyped heavy chains mutational status in CLL developing AIHA	(75)
AIHA primary/CLL and ITP	CTLA-4 exon 1	110	PCR	Pathogenic/prognostic/ therapeutic	CTLA-4 signaling is defective in AIHA, particularly in CLL cases	(76)
AIHA in CLL	miRNA−19a,20a,29c,146b-5p,186,223,324-3p,484,660	n.a.	RT-PCR	Pathogenic	Nine miRNA are preferentially expressed in CLL developing AIHA	(77)
AIHA in CLL	HCDR3 subset #3	585	PCR	Pathogenic/prognostic/ therapeutic	Sterotyped B-cell receptor subsets correlate with AIHA development	(78)
PRIMARY AND SECONDARY EVANS' SYNDROME						
Evans in CLL	IGHV	25	PCR	Pathogenic/prognostic	Majority of ES-CLL cases display stereotyped B cell receptor	(79)
AIHA and ITP	Fc-γ-R IIa and IIIa on red pulp macrophages	82	CFM and mRNA transcript analysis	Pathogenic/therapeutic	Spleen red pulp macrophages display distinct FC-γ-R expressions	(80)
AIHA and Evans in CLL	miR-150 and c-Myb	35	RT-PCR	Pathogenic	c-Myb expression is high and miR-150 is low in active hemolysis and correlate with Hb, bilirubin, and C3 levels	(81)
Pediatric Evans Syndrome	TNFRSF6, CTLA4, STAT3, PIK3CD, CBL, ADAR1, LRBA, RAG1, and KRAS	203	Sanger sequencing in 203; targeted NGS (tNGS) of 203 genes in 69 negative at Sanger ($n = 69$); whole-exome sequencing in selected cases	Pathogenic/prognostic/ therapeutic	Majority of pediatric ES display somatic mutations found in immune-deficiencies	(82)

IGHV, immunoglobulin heavy chain variable region; +3 and +12, trisomy of chromosome 3 and 12; TNF-α, tumor necrosis factor alpha; LT-α, lymphotoxin alpha; IL-10 and -12, interleukin-10 and -12; CTLA-4, cytotoxic T-lymphocyte antigen-4; IGKV, immunoglobulin K light chain variable region; TCRG, T-cell receptor gamma; TCRB, T-cell receptor beta; miRNA, microRNA; Fc-γ-R, Fc-gamma-receptor; CFM, cytofluorimetry; PCR, polymerase chain reaction; RT-PCR, real time PCR; HCDR3, heavy chain domain region 3; ES, Evans syndrome; wAIHA and cAIHA, warm and cold autoimmune hemolytic anemia; ITP, immune thrombocytopenia; CLL, chronic lymphocytic leukemia; NGS, next generation sequencing.

by the *IGHV4-34* gene, responsible for I antigen binding (63–65). Rarely, *IGHV3* family genes may also encode anti-I cold agglutinins, in particular *IGHV3-23* and *IGKV3-20* (66, 70, 83). Concerning Ig light chain genes, the *IGKV3-20* gene and the *IGHV3-15* gene are used in most cAIHA patients and contribute to I antigen binding. From a clinical perspective, mutations in the complementarity determining region (CDR)2 and in the framework region 3 (FR3) of *IGHV4-34* correlated with lower hemoglobin levels (70), whilst those in the *IGKV3-20* CDR3 correlated with younger age at diagnosis. These findings are in line with the clonal nature of cAIHA that is currently considered a distinct lymphoproliferative disorder, with some level of bone marrow infiltration morphologically different from other non-Hodgkin lymphomas. The presence of stereotyped light chains of cAIHA may be of therapeutic interest, since anti-light chain vaccinations with *IGKV3-20* are under investigation for lymphoproliferative diseases (84).

Other studies focused on B-cell receptor configuration and its contribution to AIC development. It is known that unmutated IGHV carries a strong prognostic impact on CLL course and correlates with a higher incidence of AIC (78, 85–89). The binding of auto-antigens to unmutated CLL cells activates a signal transduction (i.e., phosphorylation of SYK and ZAP-70) promoting survival and proliferation (90). More recently, a high recurrence of stereotyped IGHV aminoacid sequences has been observed in CLL patients developing AIC (91–95). Efremov et al. (73) reported an over-representation of the *51p1 VH* gene; in other two large studies ($N = 319$ and $N = 585$), patients developing AIHA showed a more frequent expression of unmutated *IGHV1-69, IGHV3-11, IGHV4-59, IGHV4–30, IGHD2-2, and IGHJ6* genes, unfavorable [del(17)(p13) and del(11)(q23)] cytogenetics, and stereotyped HCDR3 sequences (75, 78). Finally, stereotyped B cell receptor configuration was found in 66% of CLL secondary Evans syndrome, a known severe complication defined by the association of AIHA and ITP (79).

Studies on Cell-Mediated Immunity

Since a T-cell imbalance is known to play a part in AIC development (higher Th17/T regulatory ratio, Th1 to Th2 cytokine shift, increased APC activity), other studies focused on T-cell compartment. They showed the presence of clonal T-cell populations, mainly CD8+, in about 50% of AIHA patients ($N = 33$), higher than in controls (71). Another study (76) evaluated cytotoxic T-lymphocyte antigen-4 (*CTLA-4*) gene status in patients with primary or secondary AIC (20 primary AIHA, 30 CLL-AIHA, and 60 ITP). *CTLA-4* is a negative regulator of T-cell responses and has been implicated in various autoimmune diseases (96, 97). A high prevalence of an A to G polymorphism at position 49 was found among AIHA cases, particularly in the CLL-AIHA group (73% vs. 47% in the control group), suggesting *CTLA-4* mediated T-cell imbalance in these cases. A more recent study found a significant higher frequency of lymphotoxin-α (LT-α) (+252) AG phenotype in 17 AIHA cases compared to controls (41% vs. 13%) (69). LT-α (also known as TNF-β), is involved in the regulation of cell survival, proliferation, differentiation, and apoptosis, and plays an important role in innate immune regulation and immune-surveillance (98).

Finally, it has been reckoned that AIHA clinical picture also depends on the level of the monocyte-macrophage system activation and some Authors studied FcγR subtypes expressions in various tissues in 82 AIHA cases. They found that red pulp macrophages predominantly expressed the low-affinity receptors FcγRIIa and FcγRIIIa, did not express the inhibitory FcγRIIb, and expressed very low levels of the high-affinity receptor FcγRI, compared to blood monocytes (80). This may be of therapeutic interest, given that FcγR and its signaling have recently become a target in autoimmune diseases.

Genomic Studies

The use of advanced target and non-target sequencing assays offered further insights in AIHA pathogenesis. In particular, in a study of 16 primary cAIHA, next generation sequencing of bone marrow B-cells allowed the identification of recurrent mutations of *KMT2D* and *CARD11* in 69% and 31% of cases, respectively (72). Similar mutations have also been reported in lymphomas as well as in Kabuki syndrome, a congenital disorder characterized by malformations, immune-deficiency, and development of autoimmune diseases. Loss of *KMT2D* function increases B cell proliferation, impedes class switch recombination (99), and may concur to survival of autoreactive B cells synergizing with *IGHV4-34*-encoded immunoglobulin receptor stimulation (72). *CARD11* mutations were shown to induce constitutive activation of the NF-kB pathway, similarly to what observed in diffuse large B-cell lymphoma. Evaluation of *KMT2D* and *CARD11* might be of diagnostic utility in cAIHA, and would help to distinguish it from *MYD88* mutated lymphoplasmacytic lymphoma. Genomic studies may give hints for novel therapeutic approach. In fact, histone deacetylase inhibitors, that have been used in lymphoma, myeloma and Kabuki syndrome, might have a therapeutic potential in cAIHA with *KMT2D* mutations (72, 100). Similarly, therapies targeting *CARD11* gain-of-function mutations are under investigation for B cell lymphomas and may be studied also in cAIHA (101).

Another very recent study evaluated a large series of pediatric patients with Evans syndrome by Sanger sequencing, targeted NGS, and whole exome sequencing ($N = 80$): 65% received a genetic diagnosis, 49 had a germline mutation, and 3 somatic variants. Pathogenic mutations in genes involved in primary immunodeficiencies (*TNFRSF6, CTLA4, STAT3, PIK3CD, CBL, ADAR1, LRBA, RAG1,* and *KRAS*) were found in 40% of cases, and probable pathogenic variants in 16 genes not previously reported in autoimmune disease were detected in 25%. It was already known that children with primary immunodeficiency are more prone to develop immune cytopenia, whilst in adult Evans' syndrome a primary immunodeficiency was identified in 9% of cases only (102). In the pediatric study, mutated patients showed more severe disease with higher treatment requirement (>number of therapy lines) and mortality. These data confirm that a higher genomic burden is probably involved in pediatric cases, and that it seems to have prognostic and therapeutic significance (82). For instance, patients with autoimmune lymphoproliferative syndrome (ALPS), caused by germline and somatic *TNFRSF6* mutations, are more prone to develop severe persistent hypogammaglobulinemia after rituximab treatment,

and splenectomy is contraindicated. Since rituximab is highly effective and broadly used in Evans syndrome, a prompt diagnosis of such cases is of great importance. Moreover, 36% of cases had potentially targetable mutations that will be suitable for new therapeutic approaches including rapamycin inhibitors (in ALPS or a *PIK3d* activation syndrome) (103, 104), CTLA-4 fusion protein (in *CTLA-4* and *LRBA* deficiency) (105, 106), JAK inhibitors (in patients with *JAK1* or *JAK2* mutations) (107), and calcineurin inhibitors (in patients with *NFATC1* variants) (108).

Studies on MicroRNAs

MicroRNAs (miRNAs) are small single strain RNAs mainly implied in gene expression regulation at transcriptional and post-transcriptional level. They have been associated with different clinical-biological forms of CLL and are also known to play a substantial role in autoimmunity (77). In a recent study evaluating malignant B-cells from CLL-AIHA patients, nine down-regulated miRNAs were identified (i.e., miR-19a, miR-20a, miR-29c, miR-146b-5p, miR-186, miR-223, miR-324-3p, miR-484, and miR-660), of whom two (i.e., miR-20a and miR-146b-5p) known to be involved in autoimmune phenomena. Interestingly, miR-146b-5p was shown to modulate the expression of CD80, a molecule involved in the B-T cell synapse formation and in restoring the APC capacity of CLL cells. Another miRNA, miR-150, was recently studied in 35 patients with AIHA/Evans syndrome and was found low in patients with active hemolysis compared to those in remission or with CLL-AIHA. MiR-150 negatively correlated with bilirubin values and positively with Hb and complement levels, suggesting the role of miRNAs in predicting CLL evolution and treatment response (81).

MOLECULAR ASPECTS IN PRIMARY AND SECONDARY ITP

Studies on Immunoglobulin Genes

Similarly to AIHA, first molecular studies on primary ITP showed the presence of recurrent *IGHV* gene rearrangements in autoreactive B cells (**Table 3**) (109). Roark and Colleagues, found an association with rearrangements of *IGHV3-30*, and further reports showed that IGHV30 encoded IgM and IgG anti-GPIIb autoantibodies (122–125). Interestingly, *IGHV3-30* is highly employed also in AIHA, CLL, and immunodeficiencies and this may explain the association with ITP (74, 126). In CLL patients, it has been shown that the risk of developing ITP was higher among patients with stereotyped subset #1 (*IGHV1–5-7/IGHD6–19/IGHJ4*) and #7 (*IGHV1–69 or IGHV3–30/IGHD3-3/IGHJ6*) in HCDR3 region (78). Other IGHV involved in anti-platelets autoantibodies are *VH1-02, VH1-46, VH3-21*, and *VH4-59*. Interestingly, a specific heavy- and light-chain pairing seems to be necessary to enable antibody pathogenicity (127–131). Anti-platelets autoantibodies appear to share single heavy-chain VHDJH and have undergone isotype switching (hallmark of a T-cell-dependent, antigen-driven response). These aspects are not observed in naturally occurring anti-platelet antibodies that are polyreactive IgM with little or no somatic mutation of their variable regions, and are responsible for platelets turnover. The presence of stereotyped *IGHV* asset could be of therapeutic interest in ITP, since *IGHV3-30*-targeted reagents, such as anti-idiotypic antibodies derived from mice (132, 133) or humans (125) are under evaluation (134–137).

Studies on Cell-Mediated Immunity

Th17 are known to mediate autoimmunity through the release of pro-inflammatory cytokines (IL-2/IL-17). Th17 cells response, together with Th2 (anti-inflammatory), regulatory B (Breg), and Treg cells inhibition (with decrease in IL-10/TGF-β), favor ITP persistent/chronic phase. As a matter of fact, therapy with corticosteroids, rituximab, and thrombopoietin receptor agonists have all be shown to increase Tregs and TGF-β levels (TPO agonists also increase Breg). Given the importance of these cytokine dysregulation, some Authors focused on Treg/Th17 imbalance and on cytokine genes polymorphisms. In a recent study, it has been shown that *NF-κB*-94ins/del ATTG genotype (involved in the *NLRP3* inflammasome) contributes to ITP development and to imbalanced Th17 cell response (119). Another study on *IL-17F* rs763780 polymorphism, that has been associated with IL-17 expression and activity, showed a lower prevalence in ITP cases ($N = 165$) compared to healthy controls (118). Finally, Hu et al. demonstrated that IL-17A and IL-21 are able to upregulate *STAT-1, STAT-3, STAT-5* or RAR-related orphan receptor C (*RORC*), resulting in decreased Treg/Th17 balance in newly diagnosed ITP cases. This imbalance recovered after ITP remission and was reversed by the neutralization of IL-17A or IL-21 through targeting antibodies (111). IL-21 levels, together with IL-4, were also found to be abnormal in pediatric ITP ($N = 85$), and to affect T follicular helper cells levels and regulation (116). IL-17A or IL-21 blockade could be a novel target for ITP.

Studies on Inflammatory Cytokines

Interferon (IFN)-γ signaling and tumor necrosis factor (TNF) are highly implicated in ITP pathogenesis and provides a link between autoimmunity, inflammation, and bone marrow failure. A polymorphism in the signal transducer and activator of transcription 1 protein (STAT1) rs1467199 SNP, the main target of IFN-γ down-stream emerged in a study of 328 ITP children, and was differentially found between newly diagnosed and chronic patients (112). More recently, microarray studies showed that a huge number of long non-coding RNAs (lncRNAs) were significantly up-regulated or down-regulated in newly diagnosed and chronic ITP patients vs. healthy individuals. TNF and granulocyte macrophage colony-stimulating factor signaling were the most interested pathways. Interestingly, lncRNAs ENST00000440492, ENST00000528366, NR_038920, and ENST00000552576 were able to distinguish newly diagnosed from chronic ITP (120). Finally, Peng et al. used gene expression profiling analysis and whole-exome sequencing on samples from family members with ITP, sporadic ITP cases and healthy individuals and identified a potential pathologic p.G76S heterozygous mutation on the *TNFRSF13B* gene. Mutated cases had upregulated cytokine-cytokine receptor interaction, increased serum TNFα, IL-17α, IFNγ, and BAFF levels, and enhanced binding capacity of APRIL ligand to B cells. Moreover,

TABLE 3 | Molecular findings in primary and secondary immune thrombocytopenia (ITP).

Disease	Gene/Pathway	No. of patients	Technique	Impact and significance		References
PRIMARY AND SECONDARY ITP						
ITP	IGVH3-30	2	PCR	Pathogenic/therapeutic	Anti-PLT antibodies are clonally restricted	(109)
ITP	CD41, c-Myb, c-MPL, caspase-2, caspase-9, GATA-1, Bcl-xl	Murine models	RT-PCR	Pathogenic	Hyperexpression of those genes in the spleen of ITP mice	
ITP	Haptoglobin	58	Matrix assested laser desorption/ionization time-of-flight mass spectrometry	Prognostic/predictive	High haptoglobin levels predict long-term response to splencetomy	(110)
ITP	Th17 associated signaling factors	–	–	Pathogenic	Neutralization of IL-17A and IL-21 regulates Treg/Th17 imbalance	(111)
ITP	STAT1	328	Sequenom Mass Array	Pathogenic	STAT1 rs1467199 SNP plays a role in IFN-γ dependent development of ITP	(112)
ITP	miRNA	32	RT-PCR	Pathogenic/therapeutic	44 miRNAs are differentially expressed in ITP pre- and post-QSBLE therapy	(113)
ITP	miRNA-125a-5p	30	RT-PCR	Pathogenic	lncRNA MEG3 inhibits miRNA-125a-5p favoring Treg/Th17 imbalance	(114)
Primary and secondary ITP	Proteomics	134	Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry	Diagnostic	6 marker proteins distinguishing primary from secondary ITP	(115)
ITP	Bcl-6, c-Maf, Blimp-1, ICOSL, TACI, BAFFR	85	RT-PCR	Pathogenic	T follicular helper cells display different frequency and regulation between newly diagnosed and chronic pediatric ITP	(116)
ITP	TNFRSF13B	2	GEP and WES	Pathogenic	G76S mutation is a gain-of-function mutation and predispose to familial and sporadic ITP	(117)
ITP	IL-17F rs763780	165	RT-PCR	Pathogenic	IL-17F rs763780 G allele frequency is significantly lower in ITP vs. controls	(118)
ITP	NLRP3 inflammosome	403	RT-PCR	Pathogenic/therapeutic	NF-Kb-94ins/del ATTG genotype correlates with Th17 imbalance	(119)
ITP	Long non-coding RNAs	64	Microarray studies and RT-PCR	Pathogenic	lncRNAs are differentially upregulated/downregulated in newly-diagnosed and chronic ITP vs. healthy controls	(120)
ITP	Integrated mRNA and miRNA	4	Microarray technique and RT-PCR	Pathogenic	Cellular stress response is deregulated in mesenchymal stem cells from ITP cases	(121)

ITP, immune thrombocytopenia; CLL, chronic lymphocytic leukemia; IGHV, immunoglobulin heavy chain variable region; PLT, platelets; Th17, T- helper 17 cells; Th1, T helper 1; IL-17 and -21, interleukin-17 and -21; lncRNA, long non-coding RNA; Treg, T regulatory cells; miRNA, microRNA; GEP, gene expression profiling; WES, whole exome sequencing; PCR, polymerase chain reaction; RT-PCR, real time PCR; NGS, next generation sequencing.

B cells transfected with the G76S mutation could induce human megakaryocyte apoptosis *in vitro* (117).

Studies on MicroRNAs

MiRNAs expression was also evaluated in ITP in various reports: molecular studies of bone marrow mesenchymal stem cells from ITP patients showed that 740 genes and 32 miRNAs were differentially expressed compared to controls and correlated with the presence of cellular growth defects and functional abnormalities. The latter seem to be due to impaired cellular stress response, unfolded protein response, and reduced DNA transcription (121). Burenbatu and Colleagues, identified 44 miRNAs that are differentially expressed in ITP patients before and after treatment with the Mongolian medicine Qishunbaolier (QSBLE). Interestingly, 25 from these 44 miRNAs are downregulated in ITP as compared to controls, and are restored after QSBLE exposure (113). Finally, reduced miR-125a-5p expression has been linked Treg/Th17 imbalance. Li et al. demonstrated that miR-125a-5p expression is inhibited by *MEG3* overexpression in ITP patients ($N = 30$). Interestingly,

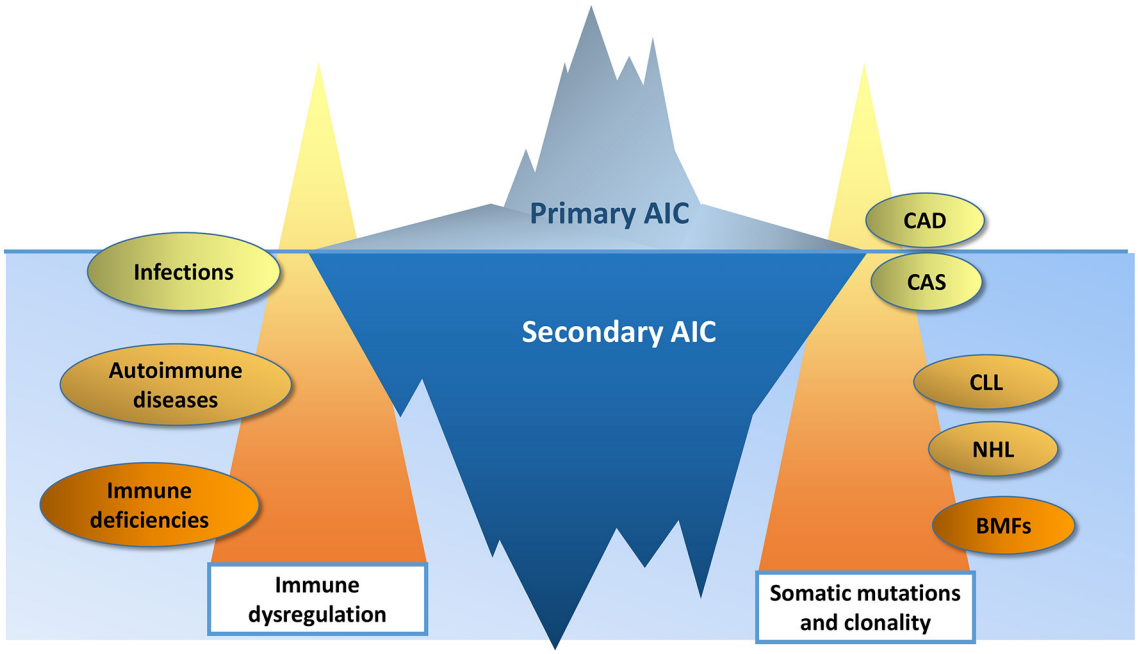

FIGURE 2 | The changing border between primary and secondary autoimmune cytopenias (AIC). Immune dysregulation is more profound in AIC secondary to systemic autoimmune diseases and immune deficiencies, than in AIC secondary to infections. Likewise, a higher burden of somatic mutations is more typical of bone marrow failures (BMF) and lymphoproliferative disorders (chronic lymphocytic leukemia, CLL; non-Hodgkin lymphomas, NHL), than in cold agglutinin disease (CAD) and syndrome (CAS). The increasing availability of genomic testing will improve the diagnostic sensitivity, moving upward the border between primary and secondary AIC.

dexamethasone was able to reduce *MEG3* expression *in vitro*, thus restoring Treg/Th17 ratio (114).

Proteomics

Proteomic studies found some clinical implications: screen of 64 primary and 70 secondary ITP cases using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) allowed the identification of 6 proteins able to distinguish primary from secondary cases with high sensitivity (115). Another proteomic study identified higher haptoglobin levels as a favorable serum biomarker for predicting long-term response to splenectomy in ITP, with a positive correlation with postoperative platelet count (110).

DISCUSSION AND FUTURE PERSPECTIVES

AIC secondary to CLL are a nice model of close intersection between cancer and autoimmunity. Both are the result of uncontrolled and dysregulated homeostatic mechanisms leading to aberrant proliferation and activity of specific cellular subsets with heterogeneous epiphenomena. Leukemic B-cells show impaired apoptosis, are unable to efficiently produce immunoglobulins, may function as antigen presenting cells, and release a variety of inflammatory cytokines leading to three main immune-related complications: infections, autoimmune diseases, and decreased immune-surveillance on secondary malignancies. These complications seem to correlate with advanced stage CLL and with poor prognostic markers. Moreover, CLL therapy may have an impact on their development.

The genomic landscape of primary and secondary AIC is of particular interest, since the type and the depth of the immune response is likely under genetic control and it could be hypothesized that a predisposing genetic background correlates with a more profound immune dysregulation. Molecular studies performed so far, mainly focused on B-cell/autoantibodies characteristics and functioning, and on T cell aberrations: sterotyped B cells with specific IGHV and light chain configuration are involved in AIC development, clonal T cells, specifically CD8+ ones are present, and various cytokine genes polymorphisms may correlate with Treg/Th17 imbalance. Other experiences showed a dysregulation at the gene expression level as demonstrated by altered miRNA and lnRNA profiles in AIC cases compared to healthy subjects, but also in newly-diagnosed vs. chronic patients, and in the same patients in different tissues. Finally, proteomic studies reported differentially translated proteins in primary vs. secondary cases. In this regard, all the guidelines on AIC state that secondary causes should always be excluded. However, current workup relies mainly on laboratory, morphologic and imaging techniques that could be unable to disclose the presence of clonal disorders (**Figure 2**). In this context, the genetic/molecular characterization of AIC patients will probably increase our sensitivity in diagnosing secondary cases. This has been demonstrated in the recent paper on a pediatric Evans' population, where NGS/WES techniques

revealed the presence of an underlying disease in 65% of cases, with important clinical/therapeutic implications. No data are available for adults, but for cAIHA, where a clonal lymphoid infiltrate is almost invariably present. This form is particularly difficult to distinguish from secondary cases. Berentsen and Colleagues proposed to differentiate cold agglutinin "disease" from "syndrome" basing on the absence or presence of a secondary cause. The demonstration that *MYD88* mutation is always absent and that *KMT2D* and *CARD11* ones are present in a proportion of cases, carry diagnostic, prognostic and therapeutic impact, further stressing the utility of molecular studies in AIC. Finally, there is growing evidence that AIC may evolve to overt clonal diseases of myeloid or lymphoid lineages and no predictors are available (138–141). This tempts to speculate about a model of "double clonality" unique for these forms, where either myeloid or lymphoid populations may undergo clonal expansion/selection. As a matter of fact, clonality and malignancy are distinct although overlapping concepts, and the evolution of a clonal disorder into an overt malignancy may require a long time, even longer than human lifespan. The immune system has a role in this process. However, it is not always clear whether it acts as an effector or spectator, and the exact molecular/genetic mechanisms and therapeutic implications have still to be disclosed.

AUTHOR CONTRIBUTIONS

BF and WB designed and wrote the review and participated to the final revision. All authors participated to the design of the review, literature revision, manuscript writing, and final revision for important intellectual content.

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Clinical Applications and Future Directions of Minimal Residual Disease Testing in Multiple Myeloma

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In the last years, the life expectancy of multiple myeloma (MM) patients has substantially improved thanks to the availability of many new drugs. Our ability to induce deep responses has improved as well, and the treatment goal in patients tolerating treatment moved from the delay of progression to the induction of the deepest possible response. As a result of these advances, a great scientific effort has been made to redefine response monitoring, resulting in the development and validation of high-sensitivity techniques to detect minimal residual disease (MRD). In 2016, the International Myeloma Working Group (IMWG) updated MM response categories defining MRD-negative responses both in the bone marrow (assessed by next-generation flow cytometry or next-generation sequencing) and outside the bone marrow. MRD is an important factor independently predicting prognosis during MM treatment. Moreover, using novel combination therapies, MRD-negative status can be achieved in a fairly high percentage of patients. However, many questions regarding the clinical use of MRD status remain unanswered. MRD monitoring can guide treatment intensity, although well-designed clinical trials are needed to demonstrate this potential. This mini-review will focus on currently available techniques and data on MRD testing and their potential future applications.

Keywords: multiple myeloma (MM), minimal residual disease (MRD), clinical practice, next-generation flow (NGF), next-generation sequencing (NGS), PET/CT

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INTRODUCTION

The treatment course of multiple myeloma (MM) has been strongly improved during the last 20 years: the introduction of modern 3-drug regimen therapies combined with transplantation increased the achievement of deeper responses and the acquisition of minimal residual disease (MRD) negativity in up to 40/50% of patients enrolled in clinical trials (1). Consistently, a large number of studies showed that, among patients achieving a complete response (CR), those with detectable MRD had inferior progression-free survival (PFS), and overall survival (OS) compared to those with undetectable MRD. Moreover, among patients in CR, improved PFS and OS have been significantly associated with undetectable MRD, regardless of disease stage, prior transplant, or cytogenetic risk (2).

Therefore, the International Myeloma Working Group (IMWG) recently revised the response criteria and introduced the definition of MRD in CR patients as the persistence or re-emergence of very low levels of cancer cells, equal to about 1 tumor cell in at least 10^5 normal cells (3). These response criteria are the direct result of the progressive evolution of both imaging and bone marrow

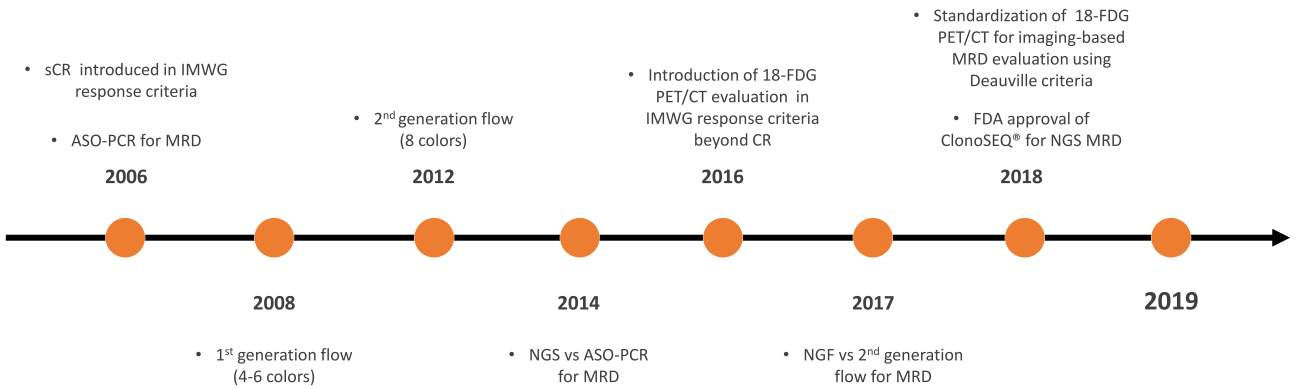

FIGURE 1 | Timeline on the development and validation of MRD techniques in MM. MRD, minimal residual disease; MM, multiple myeloma; CR, complete response; sCR, stringent CR; IMWG, International Myeloma Working Group; ASO-PCR, allele-specific oligonucleotide polymerase chain reaction; NGS, next-generation sequencing; 18-FDG PET/CT, 18-fluorodeoxyglucose positron emission tomography/computed tomography; ClonoSEQ®, ClonoSEQ® Assay (Adaptive Biotechnologies, Seattle, US-WA); FDA, Food and Drug Administration.

TABLE 1 | Bone marrow techniques for MRD in myeloma: pros and cons.

	Next-generation flow (NGF)	Next-generation sequencing (NGS)
Applicability	Nearly 100%	≥90%
Availability	Many laboratories with 4–6 colors; >8 colors restricted to more specialized centers	Commercial service only; ongoing efforts by academic platforms
Diagnostic sample	Not required	Required for identification of dominant clonotype
Number of cells required	10 million cells/tube	1–2 million cells/20 μg DNA
Sample processing	Requires a fresh sample; assessment within 24–48 h	Can use both fresh and stored samples
Standardization	EuroFlow consortium	Commercial companies (Adaptive Biothcnologies) Academic methodologies also available
Sample quality control	Possible to check by global bone marrow cell analysis	Not possible
Quantitative	Yes	Yes
Sensitivity	1 in 10^{-5}-10^{-6}	1 in 10^{-5}-10^{-6}
Turnaround and complexity	3–4 h. Requires flow cytometry skills. Automated software available	1 week. Academic methodologies require bioinformatics support
Clonal evolution	Not evaluable	Evaluable: can take into account all minor clones

MRD, minimal residual disease.

MRD techniques in the last 15 years (**Figure 1**). However, a precise knowledge of when and how to perform MRD detection is required. This review aims to examine the currently available MRD techniques recommended by IMWG and data from different clinical trials, in order to outline a possible future perspective on the role of MRD testing as a tool for decision making in standard clinical practice.

MRD TECHNIQUES AND PRACTICAL CONSIDERATIONS

Bone Marrow Techniques: NGF and NGS

There are two techniques commonly used to detect MRD in the bone marrow (BM): multiparameter flow cytometry (MFC) and next-generation sequencing (NGS) molecular technology. Both techniques show positive and negative aspects (**Table 1**).

MFC can detect and quantify tumor vs. normal plasma cells using cell surface and cytoplasmic markers. For the identification of plasma cells, the combined use of CD38 and CD138 is recommended even if they are also expressed on other BM cells. In particular, the aberrant expression patterns of CD19, CD56, CD45, CD38, CD27, CD20, CD28, CD33, CD117 and surface membrane immunoglobulin can characterize the phenotype of monoclonal plasma cells (4). However, antigenic expression can vary on plasma cells and should be considered when interpreting flow data.

Older conventional 4- to 7-color flow cytometry assays have now been replaced by advanced 8-color 2-tube or 10-color 1-tube assays. In this sense, the increased sensitivity of MFC (between 10^{-4} and 10^{-5}) is due to the simultaneous assessment of ≥8 markers in a single tube. In this way, if sufficient cell numbers are evaluated (e.g., $\geq 5 \times 10^6$), it is possible to promptly identify aberrant PC phenotypes at MRD levels (5).

A consensus methodology has been recently proposed by the International Myeloma Foundation's Black Swan Research Initiative, which formed the EuroFlow Next-Generation Flow (NGF) panel in order to increase sensitivity and standardization of MFC (6, 7). This panel includes two 8-color tubes (tube 1: CD138, CD27, CD38, CD56, CD45, CD19, CD117, CD81; tube 2: CD138, CD27, CD38, CD56, CD45, CD19, cIgκ, cIgλ). In this protocol, a bulk-lysis procedure was determined and the acquisition of $\geq 10^7$ cells/sample was recommended. Importantly, new softwares were developed for automatic plasma cell gating in order to avoid individual assessments.

According to this consensus methodology, it is important to evaluate the limit of quantitation (LOQ) and the limit

of detection (LOD) of the NGF-MRD method. The LOQ is calculated as 50 among 10^7 nucleated cells (based on the identification of ≥50 clonal plasma cells); the LOD as 20 among 10^7 nucleated cells (based on the identification of ≥20 clonal plasma cells). This evaluation allows to discriminate between positive and negative samples. Interestingly, a baseline sample is not mandatory for MRD evaluation. After the multicenter evaluation of patients with very good partial response (VGPR) or CR, 110 follow-up bone marrows showed a higher sensitivity for NGF-MRD, as compared to conventional 8-color flow-MRD: MRD-positive rates were 47 vs. 34% ($P = 0.003$), respectively. Thus, 25% of patients who were categorized as MRD-negative by conventional 8-color flow were categorized as MRD-positive by NGF. This translated into a significantly longer PFS using NGF to discriminate between MRD-negative and MRD-positive CR patients ($P = 0.02$). Importantly, NGF can also provide a qualitative assessment of the patient sample by allowing the complete analysis of the normal B-cell compartment and the detection of a significantly decreased number of non-PC BM cells (e.g., mast cells, nucleated red blood cells, myeloid precursors, B-cell precursors, and CD19– normal PC) revealing potentially hemodiluted BM samples. Finally, treatment with CD38 antibodies such as daratumumab and isatuximab can alter the antigen expression in MM cells. This sets a limit for the use of CD38 as a marker for the detection of plasma cells during MRD assessments at follow-up. The use of multi-epitope CD38 antibody in an advanced flow cytometry panel can solve this problem, since this conjugate can bind to a specific site (not covered by daratumumab) of the CD38 antigen. Nonetheless, in case of CD38 surface downregulation, the solution is the analysis of intracellular CD38 through the same protocol used for intracellular k- and λ-chain staining (7).

Allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) was first explored to evaluate molecular MRD in MM, but even if its prognostic role was confirmed, different issues limited its use in favor of the NGS technique. First, its applicability ranged from 40 to 60% due to the low rate of diagnostic marker identification, since this technique does not take into account the somatic hypermutation rate of immunoglobulin loci and this translates into sequencing problems. Moreover, patient-specific reagents raised the complexity of this technique (8–11).

NGS was developed to overcome all these disadvantages. ClonoSEQ® Assay (Adaptive Biotechnologies, Seattle, US-WA) is the most frequently adopted commercial platform in the United States. In this test, DNA is extracted from patient's BM, a multiplex PCR amplifies VDJ, IgK, and IgL gene sequences and a common PCR prepares DNA for sequencing and creates a sequencing library. At the end of the process, a bioinformatic tool is essential to extrapolate and analyze all NGS data.

Using this assay, we can define as "clonotypes" two identical sequencing reads. A clonotype with frequency >5% at diagnosis is considered a clonality (clonal gene rearrangements), thus becoming a target for the detection of MRD in follow-up samples (12, 13). In lymphoid malignancies, NGS and ASO-PCR have been compared, showing similar sensitivities and results (13).

In the IFM2009 clinical trial, a comparison between NGS and 7-color MFC has been made, showing that the higher sensitivity with NGS at 10^{-6} allowed to predict the best outcomes in MRD-positive vs. -negative patients (3-year PFS: 53 vs. 83%, $p < 0.001$).

Ongoing clinical trials are evaluating NGS vs. NGF and their correlation: in the CASSIOPEIA trial, a good concordance (83.5% in paired samples) was observed using the same sensitivity (10^{-5}) regardless of response in patients achieving ≥CR, indicating that both techniques performed similarly in evaluating MRD (14). As illustrated in **Table 1**, some characteristics can affect the clinician's preference of choosing NGS vs. NGF, such as the higher cost for NGS (∼1,500 $ per sample vs. ∼300 $ for NGF), and the required time and skills (at least 1 week for NGS vs. 3–4 h for NGF and commercial service available only for NGS).

In this regard, ongoing studies are evaluating 'in-house' NGS techniques: recently, Martinez-Lopez et al. described a NGS method starting from 1 μg of DNA and amplified IGH or IGK sequences. The sequencing data were analyzed by specific mathematical and bioinformatic tools to identify and quantify the clonotype present on each sample. A clonotype was identified when at least 400 identical sequencing reads were obtained, or when it was present at a frequency of >1% with a sensitivity of at least 10^{-5} (15).

Imaging Techniques: PET/CT

MM is a patchy disease and BM infiltration may often be heterogeneous. Indeed, ∼60% of MM patients show focal lesions that represent the local accumulation of plasma cells (16). Therefore, the IMWG incorporated imaging in addition to BM evaluation to better characterize MM residual disease (3).

Different studies showed the role of imaging techniques in evaluating focal lesions: magnetic resonance imaging (MRI) is a sensitive, non-invasive imaging technique available to detect the bone involvement in the spine and to provide details regarding the soft tissue disease and the pattern of marrow infiltration (normal, focal, diffuse, or heterogeneous).

Fluorodeoxyglucose positron emission tomography/computed tomography (FDG PET/CT) can be used to analyze the vitality of the focal lesions and is therefore the current standard of care to evaluate the post-therapeutic residual infiltration (17–19).

Different studies showed the prognostic and predictive role of FDG PET/CT (20–22). Interestingly, Moreau et al. compared PET/CT with MRI. Although at diagnosis both the techniques performed similarly in the detection of bone lesions, the normalization after therapy of PET/CT, but not of MRI, was predictive of PFS and OS (20). In both responding and non-responding patients, focal lesions can still remain positive for many months. As a consequence, conventional MRI is probably not the best technique to evaluate MRD (22–24). On the other hand, functional MRI techniques based on the measurement of the movement of water molecules in the tissue (Diffusion-Weighted MRI, DWI) could be informative on the residual cellularity and the microcirculation of the focal lesions (25). No standardization of the diagnostic technique and no interpretation of results in MM after therapy are still available and no prospective comparison between PET/CT and DWI in

a meaningful number of patients has been done. In a small number of MM patients, DWI seemed to be more sensitive in the detection of residual lesions. However, if this could be an advantage or could lead to an increased number of false-positive cases, still needs to be elucidated (26, 27).

Finally, different researchers confirmed the complementarity of PET/CT and BM techniques. Rasche et al. showed how patients who were both Flow-MRD- and PET/CT-negative had the best PFS outcome when compared with those who were Flow-MRD-negative but PET/CT-positive (28). Paiva et al. demonstrated that, even if NGF-negative patients had a long PFS, there was a proportion of subjects who relapsed with extramedullary disease in the presence of a previous negative BM sample, confirming the importance of combining BM and imaging analyses (29).

PET/CT has some limitations, some of which are linked to the tracer used (FDG). Indeed, a low expression of the enzymes responsible for the glycolysis process (e.g., hexokinase 2 gene) in MM cells could lead to false-negative cases with FDG PET/CT (30). Alternative tracers could overcome these limitations. For instance, 11C-Methionine uptake correlates with protein synthesis, a very active mechanism in malignant plasma cells, and can be used as an alternative PET/CT tracer in MM (31).

In a head-to-head prospective comparison in a heterogeneous MM patient population, 11C-Methionine PET/CT was more sensitive than FDG PET/CT in the detection of focal lesions, both within and outside the bone. More data are needed in a homogenous patient population to understand whether this tracer could be an alternative to FDG in the detection of residual disease after treatment. Currently, other tracers targeting lipid membrane (e.g., Choline, Acetate) and CXCR4 are also under study (32).

MRD RESULTS IN THE CLINICAL SETTING: RELEVANT QUESTIONS

In this section we focus on clinically relevant questions regarding MRD, reviewing available data on newly diagnosed MM (NDMM) patients. Single studies are summarized in **Table 2**. Data on MRD evaluation in relapsed and/or refractory MM patients (59) and high-risk smoldering MM (60) are beginning to emerge as well, and have been recently reviewed elsewhere (61).

In the MM field, a major question concerned the prognostic role of MRD and its ability to perform better than conventionally defined response criteria. As already discussed, there is now compelling evidence coming from multiple studies (**Table 2**) and two meta-analyses (2, 62) confirming that MRD-negative patients have a significantly better PFS and OS compared to MRD-positive patients. The beneficial effect of MRD negativity was confirmed also focusing on CR patients (2). Using MFC with a sensitivity of 10^{-4}-10^{-5}, Lahuerta et al. nicely demonstrated that MRD-negative patients with a conventionally defined CR had better PFS (median, 63 vs. 27 months, $p < 0.001$) and OS (median, not reached vs. 59 months, $p < 0.001$) than MRD-positive CR patients (42). Moreover, MRD-positive CR patients had similar outcomes compared to patients achieving a partial response (PR) (median PFS, 27 vs. 29 months; median OS, 59 vs. 65 months, respectively) showing that the prognostic advantage of conventionally defined CR over PR resided in the MRD-negative patient population (42).

The best timing for MRD measurement is another important unanswered question. Usually, MRD is measured at specific timepoints during therapy [e.g., *post-induction* (39), *+100 days post-ASCT* (33), *post-consolidation* (41), *pre-maintenance,* and *during maintenance* (46)]. If treatment does not provide for a phase-specific timepoint (as in the case of the continuous treatment strategy commonly adopted for transplant-ineligible patients), MRD testing is usually done at unconfirmed CR/sCR and at fixed timepoints thereafter (50).

Data clearly show that, as we continue to intensify patient treatment, the percentage of MRD-negative patients increases (39, 43, 53, 55, 56) and even maintenance treatment can convert a significant percentage of MRD-positive patients into MRD-negative [e.g., 27–30% with lenalidomide maintenance in a pooled analysis (9, 46)]. Each timepoint can be important due to different clinical reasons. For instance, the *post-induction* timepoint can be used to design clinical trials addressing different intensification regimens, while *pre-maintenance* or *during maintenance* timepoints can be exploited to design clinical trials addressing the intensity and the duration of maintenance. Regarding the prognostic effect of different timepoints, in the Myeloma IX study, which used MFC with a sensitivity of 10^{-4}, a PFS advantage was found in patients that were MRD-negative both *post-induction* and *post-ASCT*, as compared with patients that were MRD-positive *post-induction* and became MRD-negative *post-ASCT*, although this effect did not translate into an OS benefit (34). On the other hand, Hahn et al. demonstrated in a transplant-eligible population that patients who were MRD-negative *pre-ASCT*, *pre-maintenance,* and *1-year post-ASCT* showed all a better PFS compared to MRD-positive patients. Only the *1-year post-ASCT* timepoint was associated with better OS (3-year post-ASCT OS 96 vs. 66% for MRD-negative vs. MRD-positive patients) (53). These data suggest that the duration of MRD negativity may be important, but little data are available on sustained MRD negativity (i.e., the need to confirm MRD at different timepoints) and on its optimal duration. Gu et al. used MFC to monitor 104 MM patients *post-induction* and at different *post-ASCT* timepoints (3 to 24 months), showing that patients with persistent MRD negativity *post-induction* until 24 months after ASCT ($n = 33$) had better time to progression (median, not reached vs. 15.4 months) and OS (not reached vs. 35.2 months), as compared to patients that were MRD-negative *post-induction* but MRD-positive within 24 months *post-ASCT* ($n = 5$) (43). The low numbers in the latter group do not allow the exploration of different time cutoffs for sustained MRD negativity. However, 2/5 patients became MRD-positive 18 months *post-ASCT*, thus suggesting that long-term confirmation of sustained MRD negativity may be necessary.

Another question is whether the sensibility of the technique impacts the reliability of MRD. Using MFC with a sensibility of 10^{-4}, Rawstron et al. demonstrated that each log depletion in MRD levels predicted a 1-year median OS advantage (5.9 years for 10^{-2}-10^{-3}, 6.8 years for 10^{-3}-10^{-4}, and more than 7.5

TABLE 2 | Selected trials on NDMM patients reporting MRD data.

Study	Patient population[a] (*n*)	Method (sensitivity)	Timepoint	MRD-negative[b] (%)	Outcomes (MRD neg vs. MRD pos)
Paiva et al. (33)	TE NDMM in ≥PR after 6 alternating VBMCP/VBAD cycles and ASCT (295)	MFC (10^{-4})	+100 days after ASCT	42%	Median PFS: 71 vs. 38 months
Rawstron et al. (34)	NDMM: - Intensive arm (378): CTD (178) or CVAD (190) induction + ASCT. - Non–intensive arm (245): MP (119) or aCTD (126).	MFC (10^{-4})	Post-induction (both arms) and +100 days after ASCT (intensive arm only)	Intensive arm: Post CTd induction 25% (71% post-ASCT) Post CVAD 13% (54% post-ASCT) Non-intensive arm: Post MP induction 3% Post aCTd induction 26%	Intensive arm: Median PFS* according to post-ASCT timepoint 29 vs. 16 months Non-intensive arm: Median PFS* according to post-induction timepoint 10.5 vs. 7.4 months
Puig et al. (10)	NDMM in ≥PR (102)	ASO-PCR (10^{-4})	Post-induction (NTE patients) or +100 days after ASCT (TE patients)	46%	TE patients: median PFS 54 vs. 27 months NTE patients: median PFS NR vs. 31 months
Kumar et al. (35)	NDMM receiving IRd induction + ixazomib maintenance (64)	MFC (10^{-4})	Mostly at suspected CR	12.5%	NA
de Tute et al. (36)	NTE NDMM after aCTD or aRCD induction (297)	MFC (10^{-4})	Post-induction	aCTD arm: 11% aRCD arm: 16%	aCTD arm: median PFS 34 vs. 19 months aRCD arm: median PFS 32 vs. 17 months
Ludwig et al. (37)	TE NDMM in CR after 4 cycles of VTd or VTd+cyclophosphamide induction and ASCT (42)	MFC (not specified)	Suspected CR	81%	Median PFS NR vs. 39 months
Paiva et al. (38)	NTE NDMM in ≥PR after 6 VMP (52) or VTP (50) induction cycles	MFC (10^{-4}-10^{-5})	Post-induction	30%	3-year PFS: 90% vs. NR
Roussel et al. (39)	TE NDMM after 3 VRd + ASCT + 2 VRd cycles followed by lenalidomide maintenance (31)	MFC (10^{-4}-10^{-5})	Longitudinal	Post-induction: 16% Post-ASCT: 54% Post-consolidation: 58% Post-maintenance: 68%	3-year PFS according to post-maintenance MRD: 100% vs. 23%
Paiva et al. (40)	TE NDMM in ≥CR after ASCT (241)	MFC (10^{-4}-10^{-5})	+100 days after ASCT	64%	3-year TTP: 76% vs. 58%
Ferrero et al. (41)	TE NDMM in ≥VGPR after ASCT (39) undergoing VTd consolidation	ASO-PCR (10^{-4}-10^{-5})	Longitudinal	Post-ASCT: 23%, Post-consolidation: 57% 6-month post-consolidation: 72%	Median PFS: 68 vs. 23 months
Korthals et al. (11)	TE NDMM after 2–4 cycles of idarubicin-dexamethasone undergoing ASCT	ASO-PCR (10^{-4}-10^{-5})	Post-induction and post-ASCT (+3–6 months)	Post-induction: 17% Post-ASCT: 21%	NA
Lahuerta et al. (42)	NDMM alive and with MRD data available at 9 months after treatment start (609)	MFC (10^{-4}-10^{-5})	9 months after treatment start	43%	Median PFS* 63 months vs. NA (11–29 months in the other response categories)
Gu et al. (43)	TE NDMM (101)	MFC (50^{-4}-10^{-5})	Longitudinal	Post-induction: 37% Post-ASCT: 66% 2-year post-ASCT: 78%	Median TTP: NR vs. NR
Korde et al. (44)	NDMM receiving 8 KRd induction cycles (45)	NGS (not specified)	Post-induction	42% (calculated on NGS-evaluable NDMM patients)	18-month PFS: 100% vs. 84%
Martin-Lopez et al. (45)	NDMM in ≥VGPR (121)	NGS [10^{-5}]	Post-induction (NTE patients) or +100 days after ASCT (TE patients)	27%	Median TTP: 80 vs. 31 months
Oliva et al. (46)	TE NDMM in ≥VGPR after consolidation (73) followed by lenalidomide maintenance	ASO-PCR (10^{-5})	Pre-maintenance and during maintenance	Pre-maintenance: 45% During maintenance: 60%	Median PFS: NR vs. 48 months
Oliva et al. (47)	TE NDMM in ≥VGPR after VCd induction, VMP vs. ASCT intensification, VRd vs. no consolidation (316) followed by lenalidomide maintenance	MFC (10^{-5})	Pre-maintenance and during maintenance	Post-consolidation: 76%	3-year PFS*: 77% vs. 50%
Paiva et al. (48)	NTE NDMM with response (80% of the patients with ≥VGPR) after 18 sequential or alternating VMP/Rd cycles (162)	MFC (10^{-5})	After 9 cycles or 18 cycles	Sequential arm 9-cycles: 20% 18-cycles: 46% Alternating arm 9-cycles: 19% 18-cycles: 33%	Median TTP*: NR vs. 15 months

(Continued)

TABLE 2 | Continued

Study	Patient population[a] (*n*)	Method (sensitivity)	Timepoint	MRD-negative[b] (%)	Outcomes (MRD neg vs. MRD pos)
Mateos et al. (49)	NTE NDMM: DaraVMp arm (350) - VMp arm (356)	NGS (10^{-5})	Longitudinal	- Dara-VMp arm: 22.3% - VMp arm: 6.2%	NA
Facon et al. (50)	NTE NDMM: - DaraRd arm (368) - Rd arm (369)	NGS (10^{-5})	Longitudinal	Dara-Rd: 24.2% Rd arm: 7.3%	NA
Voorhees et al. (51)	TE NDMM receiving Dara-VRd induction, ASCT and Dara-VRd consolidation (13)	NGS (10^{-5})	Longitudinal	Post-induction: 19% Post-consolidation: 50%	NA
Gay et al. (52)	TE NDMM receiving KCd-ASCT-KCd (arm A, 159), KRd-ASCT-KRd (arm B, 158), 12 cycles of KRd (arm C, 157)	MFC (10^{-5})	Pre-maintenance	Arm A: 42% Arm B: 58% Arm C: 54%	NA
Flores-Montero et al. (7)	NDMM or RRMM patients achieving ≥VGPR (79)	NGF (10^{-5}-10^{-6})	Post-induction, during maintenance or post-treatment	47%	Time to 75% PFS event*: NR vs. 10 months
Hahn et al. (53)	NDMM receiving induction and ASCT ± VRd consolidation (293) followed by lenalidomide maintenance	MFC (10^{-5}-10^{-6})	Longitudinal	Pre-ASCT 42% Post-ASCT ± consolidation 78% 1 year post-ASCT 84%	Pre-ASCT 3-year PFS*: 69% vs. 60% Post-ASCT ± consolidation 3-year PFS*: 75 vs. 59% 1-year post-ASCT 3-year PFS*: 76% vs. 44%
Ocio et al. (54)	NTE NDMM receiving Isa-VRd induction + Isa-Rd maintenance (16)	NGF (10^{-5}) and NGS (10^{-5})	Longitudinal	NGF 44% (18% at 10^{-6}) NGS 50% (33% at 10^{-6})	NA
Zimmermann et al. (55)	TE NDMM receiving 4 cycles of KRd induction-ASCT-4 cycles of KRd consolidation and 10 cycles of KRd extended consolidation (76)	MFC (10^{-4}-10^{-5}) and NGS (10^{-6})	Longitudinal	MFC Post-consolidation (cycle 8) 82% Post-extended consolidation (cycle 18) 90% NGS Post-consolidation (cycle 8) 66% Post-extended consolidation (cycle 18) 71%	According to cycle 8 MRD status by MFC and/or NGS 2-year PFS: 100 vs. 93%
Avet-Loiseau et al. (56)	NDMM receiving DaraVTd-ASCT-DaraVTd (543) or VTd-ASCT-VTd (542)	MFC (10^{-5}) and NGS (10^{-6})	Post-induction Post-consolidation	Post-induction (MFC) Dara-VTd arm: 35% VTd arm 23% Post-consolidation (MFC) Dara-VTd arm: 64% VTd arm 44% Post-consolidation (NGS in evaluable patients) Dara-VTd arm: 39% VTd arm 23%	NA
Takamatsu et al. (57)	NDMM in ≥VGPR after ASCT (51)	NGS (10^{-6})	Post-ASCT (day 24–2,808)	51%	4-year PFS: 96% vs. NR
Perrot et al. (58)	TE NDMM after 8 VRd cycles or 3 VRd + ASCT + 2 VRd cycles followed by lenalidomide maintenance (509)	NGS (10^{-6})	Pre- or post-maintenance	VRd alone arm: 20% ASCT arm: 30%	Median PFS: NR vs. 29 months

[a] *If data come from a heterogeneously treated population, information about treatment is not showed. If data come from a single randomized trial, treatment data are provided.*

[b] *If data at different sensitivity levels are available, the MRD rates at highest sensitivity levels are provided.*

**time-to-event calculated from MRD assessment.*

NDMM, newly diagnosed multiple myeloma; MRD, minimal residual disease; CR, complete response; VGPR, very good partial response; MFC, multiparametric flow cytometry; ASCT, autologous stem-cell transplantation; TTP, time-to-progression; PFS, progression-free survival; ASO-PCR, allele-specific oligonucleotide polymerase chain reaction; TE, transplant-eligible; NGS, next-generation sequencing; NR, not reached; NA, not available; NTE, transplant-ineligible; Dara, daratumumab; Ixa, ixazomib; Rd, lenalidomide, dexamethasone; VRd, bortezomib, lenalidomide, dexamethasone; IRd, ixazomib, lenalidomide, dexamethasone; VTd, bortezomib, thalidomide, dexamethasone; VMP, bortezomib, melphalan, prednisone; VCd; bortezomib, cyclophosphamide, dexamethasone; KRd, carfilzomib, lenalidomide, dexamethasone; KCd, carfilzomib, cyclophosphamide, dexamethasone; CRd, cyclophosphamide, lenalidomide, dexamethasone; PR, partial response; VBMCP, vincristine, carmustine, melphalan, cyclophosphamide, prednisone; VBAD, vincristine, carmustine, adriamycin, dexamethasone; CTD, cyclophosphamide, thalidomide, dexamethasone; RCD, lenalidomide, cyclophosphamide, dexamethasone; CVAD, cyclophosphamide, vincristine, doxorubicin, dexamethasone; MP, melphalan and prednisolone; aCTD/aRCD, attenuated CTD/RCD; NGF, next-generation flow.

years for 10^{-4}), suggesting that MRD level is a continuous rather than a discrete variable (63). Recently, several studies using both flow cytometry-based methods with a sensitivity of 10^{-5} (48) or 10^{-5}-10^{-6} (7) and NGS-based methods with a sensitivity of 10^{-6} (58, 64) demonstrated that lower levels of MRD are associated with better outcomes and that the best possible sensitivity should be pursued. Indeed, in the IFM/DFCI 2009 trial, among 163 patients who were MRD-negative *pre-maintenance* using MFC with a sensibility of 10^{-4}, 84 (56%) were indeed MRD-positive using NGS with a sensibility of 10^{-6} (3-year PFS, 86 vs. 66% in NGS-negative vs. NGS-positive among MFC-negative patients). This is especially important in clinical trials designed to explore treatment interruption based on MRD levels because a low sensibility of the technique can lead to unacceptable risk of patients' undertreatment.

This observation leads to our last question: if MRD negativity is a major prognostic determinant, do treatment administered and baseline risk stratification matter as long as MRD negativity is achieved? Many studies demonstrated that even if a more effective regimen induced MRD negativity in a higher number of patients, the prognosis of MRD-negative patients was similar independently from treatment arm (49, 58). However, we do need MRD-driven clinical trials to determine if treatment deintensification in MRD-negative patients is feasible without worsening patient prognosis (65). In this regard, in the Myeloma IX trial, MRD-negative patients (MFC at 10^{-4}) receiving thalidomide maintenance remained in a MRD-negative state more often than patients not receiving maintenance treatment (96 vs. 68.8%, $p = 0.026$). Regarding MM patients who are at high risk according to baseline prognostic factors (e.g., high-risk cytogenetics or unfavorable Revised International Staging System score), MRD-negative patients at a low level of sensitivity (10^{-4}) still showed inferior clinical outcomes than standard-risk patients (34). Conversely, reaching MRD negativity at a sensitivity of 10^{-5}-10^{-6} seemed to overcome the inferior outcome observed in high-risk vs. standard-risk patients (48, 58). However, it should be noted that high-risk patients require highly intensive regimens in order to achieve a proper level of MRD negativity (47, 52, 55).

FUTURE PERSPECTIVES

Is MRD a Surrogate Endpoint for Drug Approval?

Improving OS and quality of life is the final aim of MM treatment. In the past years, the PFS endpoint has been used as a surrogate endpoint for OS to speed up the drug approval process. However, following the achievement of long-standing and deep responses (especially in NDMM patients), PFS is inappropriately becoming a late endpoint. MRD is considered the best candidate as a PFS/OS surrogate marker for provisional drug approval by regulatory agencies. Indeed, ClonoSEQ® Assay is now authorized by FDA (66) and MRD negativity with a sensitivity of 10^{-5} is the most common primary endpoint of new clinical trials designed for NDMM patients. However, as discussed above, continuous efforts should be exerted to define the optimal sensitivity cut-off (10^{-5} vs. 10^{-6}), the timing of evaluation and the need for a sustained MRD negativity. Moreover, safety should be closely addressed, as it was demonstrated by higher MRD (13.4 vs. 1%) but worse OS rates (HR 2.03, 95% CI 1.04–3.94) in the experimental arm of the BELLINI trial (M14-031) comparing venetoclax-Vd vs. Vd (67, 68). Moreover, in some settings, the correlation between MRD negativity rates and PFS improvement could be less clear because of technical pitfalls (e.g., early MRD evaluation after myelosuppressive treatments in hypocellular bone marrows).

How to Address Spatial Heterogeneity?

MM is a spatially heterogeneous disease and simultaneous MRD negativization inside and outside the bone marrow showed synergistic predictive values (28).

Moreover, MRD analysis within the bone marrow is done on bone marrow aspirates coming from a single random site and, in some patients, MM cells show a patchy infiltration (69). To overcome this issue and to possibly link the information on residual disease coming from both bone marrow and extramedullary sites, liquid biopsy approaches are beginning to emerge. Currently under exploration are the detections at high sensitivity levels of circulating tumor DNA (70), circulating plasma cells (71), and M protein peptides (72–74). The further optimization of the available techniques will be essential for their future success.

As an example, applying the ClonoSEQ® assay to peripheral blood ctDNA and paired BM samples, Mazzotti et al. showed that residual disease in the peripheral blood was undetectable in 69% of patients with concurrent MRD-positive bone marrow samples (70). This was mainly due to an insufficient sensibility to detect specific Ig gene rearrangements in the peripheral blood when disease burden was low in the BM (70), underlying the need to improve the technique before we can routinely exploit peripheral blood to monitor MM burden.

MRD-Driven Trials

MRD has not yet entered the clinical practice, but it represents an attractive tool to potentially guide treatment choices. To address this hypothesis, many MRD-driven trials are beginning to explore treatment intensification in MRD-positive patients after standard treatment (e.g., NCT03901963) or treatment deintensification in sustained MRD-negative patients (e.g., NCT03710603). Ongoing and future MRD-driven trials will contribute to solve the unanswered question: is it recommended to evaluate other induction cycles until the achievement of MRD negativity in patients who are MRD-positive after 4 induction cycles? Can we perform post-transplant consolidation on the basis of MRD status? Can we stop maintenance after 1 year of sustained MRD negativity?

Ongoing and future clinical trials will evaluate the definition and the role of sustained MRD-negativity in treatment decision-making. On the one hand, the achievement of a MRD-negative status does not necessarily mean that treatment should be stopped. Indeed, it should be noted that what we define as "MRD-negative" is a MRD undetectable with the current techniques, each one of them having a sensitivity limit. This means that we are not sure that the disease is eradicated even in MRD-negative cases. On the other hand,

the achievement of a MRD-positive status after treatment brings the question of whether it is necessary to change treatment, improving the depth of response. However, before developing response-adjusted treatment strategies based on MRD status—either intensifying/changing treatment for MRD-positive patients or de-escalating treatment for MRD-negative patients—we need to understand if sustained MRD negativity should be the treatment goal and to define the most appropriate timepoint for its evaluation (after 1 year or after more years).

AUTHOR CONTRIBUTIONS

SO, MD'A, MB, and AL: substantial contributions to the conception or design, acquisition, analysis, or interpretation of data, critical revision for important intellectual content, final approval of the version to be published, and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. SO, MD'A, and AL: first draft. MB and AL: supervision.

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Insights Into Genetic Landscape of Large Granular Lymphocyte Leukemia

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Large granular lymphocyte leukemia (LGLL) is a chronic proliferation of clonal cytotoxic lymphocytes, usually presenting with cytopenias and yet lacking a specific therapy. The disease is heterogeneous, including different subsets of patients distinguished by LGL immunophenotype (CD8+ Tαβ, CD4+ Tαβ, Tγδ, NK) and the clinical course of the disease (indolent/symptomatic/aggressive). Even if the etiology of LGLL remains elusive, evidence is accumulating on the genetic landscape driving and/or sustaining chronic LGL proliferations. The most common gain-of-function mutations identified in LGLL patients are on *STAT3* and *STAT5b* genes, which have been recently recognized as clonal markers and were included in the 2017 WHO classification of the disease. A significant correlation between *STAT3* mutations and symptomatic disease has been highlighted. At variance, *STAT5b* mutations could have a different clinical impact based on the immunophenotype of the mutated clone. In fact, they are regarded as the signature of an aggressive clinical course with a poor prognosis in CD8+ T-LGLL and aggressive NK cell leukemia, while they are devoid of negative prognostic significance in CD4+ T-LGLL and Tγδ LGLL. Knowing the specific distribution of *STAT* mutations helps identify the discrete mechanisms sustaining LGL proliferations in the corresponding disease subsets. Some patients equipped with wild type *STAT* genes are characterized by less frequent mutations in different genes, suggesting that other pathogenetic mechanisms are likely to be involved. In this review, we discuss how the LGLL mutational pattern allows a more precise and detailed tumor stratification, suggesting new parameters for better management of the disease and hopefully paving the way for a targeted clinical approach.

Keywords: large granular lymphocyte (LGL), T-LGL leukemia (T-LGLL), chronic lymphoproliferative disease of NK cells (CLPD-NK), STAT3, STAT5b, mutation

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INTRODUCTION

The 2017 world health organization (WHO) classification includes the large granular lymphocyte (LGL) leukemia in the category of cytotoxic T and NK cell leukemia and lymphoma. LGL leukemia is a lymphoproliferative disorder, sustained by clonal mature T or NK cells, that configures T-LGL leukemia (T-LGLL) or the chronic lymphoproliferative disease of NK cells (CLPD-NK), respectively (1). T-LGLL is the most frequent form (about 85% of cases), whereas, CLPD-NK is less represented (10% of cases) (2). A third group of rare (incidence 5%) diseases accounts for

aggressive T-LGLL and aggressive NK cell leukemia (ANKL), characterized by very poor prognosis (2). T-LGLs usually express the TCR αβ+, CD4–, CD8+ phenotype and the disease is referred to CD8+ T-LGLL. The heterogeneity of the disease is emphasized by the presence, in 10–15% cases, of a disorder sustained by TCR αβ+, CD4+, CD8+/– LGLs, defining the CD4+ T-LGLL. Beyond the expansions of T cells bearing the TCR αβ+, a minority of cases originates from TCRγδ+ cells (Tγδ LGLL) (3). In addition, some patients are characterized by a bi-phenotypical variant, identified by a concomitant T/NK cell clone, or by a switch from T to NK phenotype or *vice versa* (4).

The disease is asymptomatic in nearly 30% of cases, with lymphocytosis representing the only observed hematological abnormality (5). However, during the disease course in 60% of cases therapy is needed, mostly for cytopenia-related manifestations, symptomatic patients showing clinical features often related to neutropenia (2). Currently, no specific treatment is available for LGL disorders and the current therapy is based on immunosuppressive drugs (i.e., Methotrexate, Cyclophosphamide or Cyclosporine A) giving unsatisfying responses (6, 7).

The etiopathogenesis of LGL leukemia has not been established. A viral or autologous antigen has been claimed to trigger the initial lymphocytosis whose survival over the time is then maintained by the upregulation of several cell activating pathways (8, 9). Among these, the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling is central to direct the cell toward survival, being STAT an inducer of the transcription of many pro-survival genes (10). Supporting the role of these activatory pathways, in about 40% of patients, mutations on *STAT3* and *STAT5b* have been recognized as the most common gain-of-function genetic lesions up to now identified in LGLL patients. The resulting constitutive activation of STAT3 and STAT5b promotes an upregulation of the expression of genes that are required for cell proliferation and survival, i.e., *c-Myc*, *cyclin D1* and *cyclin D2*, *Bcl-xl*, *Mcl1,* and *survivin* (11). *STAT3* and *STAT5b* mutations have been included in the 2017 WHO LGLL classification (12).

GENETICS OF T-LGLL

STAT3 Mutations

Currently, *STAT3* mutations are the most commonly recognized genetic lesions in T-LGLL. Somatic *STAT3* mutations are preferentially located in the Src homology 2 (SH2) domain of the gene, leading to an increase of the stability of STAT3 protein dimerization that results in an enhanced transcriptional activity of pro-survival proteins (13). *STAT3* mutation is preferentially found in CD8+ T-LGLL (14) and some TCRγδ LGLL cases (15), its incidence among the entire cohort of T-LGLL ranging from 11 up to 75% based on different reports (13–26). Y640F and D661Y are the most frequent *STAT3* genetic lesions, accounting for about 60% of the recognized mutations. The remnant other less frequent mutations include both point mutations and insertion or deletions and are mostly found in SH2 domain (13–26), although some missense substitutions were described in DNA-binding and coiled coil domains [(27); **Figure 1**]. All T-LGLL patients are characterized by STAT3 activation, that is the hallmark of every T-LGLL, but a higher amount of the phosphorylated protein has been observed in cases with *STAT3* mutations (13, 14, 17). Functional studies revealed that even if in different locations, most of the reported mutations lead to a higher protein transcriptional activity and cytokine responsiveness (13, 27). Nevertheless, deep transcriptional expression studies in T-LGLL did not find significant differences that distinguish patients with and without *STAT3* mutations, which showed similar overexpression of STAT3-responsive genes (13, 17, 28, 33). These findings suggest that in patients devoid of *STAT3* mutations, other mechanisms or lesions can be responsible of the activation of STAT3 pathway.

STAT5b Mutations

Another member of STAT protein family has been reported to carry gain-of-function mutations, namely *STAT5b*. Initially discovered in only 2% of CD8+ T-LGLL, specifically found in the aggressive form of LGLL (28), *STAT5b* mutations were subsequently identified in 15–55% of CD4+ T-LGLL (14, 15, 29), and in 19% of TCRγδ LGLL (15). To date, genetic alterations discovered in *STAT5b* are all point mutations located in the SH2 and in the transactivation domain of the gene (**Figure 1**). The most recurrent mutations are N642H and Y665F, both deemed to increase the protein activity. At variance with STAT3, STAT5b activation has been observed only in cell samples carrying the mutated protein, whereas the wild type protein is unphosphorylated, similarly to healthy controls (28). Notably, functional studies and transcriptional analysis verified that N642H is able to induce a strong protein activation and to characterize patients with a peculiar gene expression distinguishing them from other patients who are not equipped with this mutation (including patients with *STAT* wild type, *STAT3* mutation and with *STAT5b* Y665F) (28).

TNFAIP3 Mutations

TNFα-induced protein 3 (*TNFAIP3*) is another gene recurrently mutated in T-LGLL, that has been found altered in 4 cases within a cohort of 39 patients (24). This gene is a tumor suppressor that encodes A20, a negative regulator of nuclear factor kappa B (NF-kB), whose mutated form likely contributes to deregulate NF-kB activity. Moreover, the association between *TNFAIP3* and *STAT3* mutations (3 out of 4 cases simultaneously carry both mutations) suggests that *TNFAIP3* lesion is not only an alternative genetic mechanism to *STAT* alterations, but rather an additional event concurring to induce the LGLL phenotype.

Other Less Frequent Gene Mutations

Through deep sequencing, some authors discovered other genes occasionally mutated in T-LGLL, many of them linked to STAT3 signaling pathway and cytotoxic T lymphocyte activation, including *PTPRT*, *BCL11B*, *PTPN14*, *PTPN23* (13, 27–29, 33–35). Consistently, a systems genetic assay showed that 5 out of 8 patients devoid of *STAT* mutations carried alterations on genes "STAT related" or connecting STAT with Ras/MAPK/ERK and IL-15 signaling, including *FLT3*, *ANGPT2*, *KDR/VEGFR2,* and

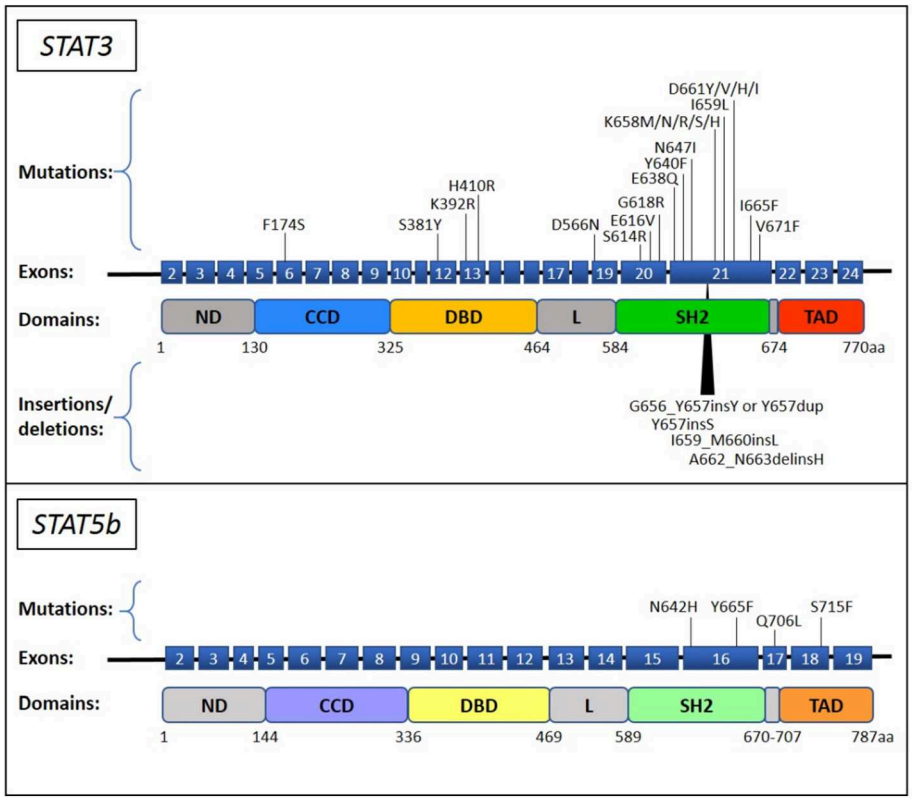

FIGURE 1 | *STAT3* and *STAT5b* mutations described in T-LGLL and CLPD-NK. The *STAT3* and *STAT5b* mutations, reported up to now in literature (13–32), are indicated on their position in the exon upstream the corresponding protein domain. The point mutations and the insertions/deletions are reported above and below the schematic representation of the gene, respectively. ND, N-terminal domain; CCD, coiled-coil domain; DBD, DNA binding domain; L, linker; SH2, Src Homology 2; TAD, transactivation domain.

CD40LG (36). These data emphasize the central role of JAK/STAT pathway in T-LGLL patients regardless of *STAT* mutational pattern. In addition, this analysis demonstrates that several genetic lesions affect different functionally connected genes that can concur to drive a similar phenotype.

GENETICS OF CLPD-NK

STAT3 and *STAT5b* Mutations

Although sharing many similarities (37), CLPD-NK is only partially reminiscent of the genetic background of T-LGLL. Since this disease is rarer than the T related entity, less data are available and appropriate analyses are lacking to precisely define this disorder. The similarity with T-LGLL involves *STAT3* gene, that is reported mutated also in CLPD-NK [(17, 38); **Figure 1**]. To note, our data indicate that *STAT3* mutations seem to have a lower incidence, from 5.9 to 8.3%, in this disease as compared to T-LGLL (15, 39). Other studies report a frequency of *STAT3* mutations from 11 up to 40%; the two studies with the largest number of CLPD-NK patients indicate 15/50 (30%) and 5/40 (13%) cases with *STAT3* mutation (17, 40).

Differently from T-LGLL, CLPD-NK appears to be devoid of *STAT5b* genetic lesions (15, 38, 39), with the only exception of the aggressive case discussed by Rajala et al., who subsequently developed ANKL (28).

Other Less Frequent Gene Mutations

In line with T-LGLL, *TNFAIP3* mutation has been found in one out of 17 CLPD-NK patients (5.9%) (38).

Other genetic alterations have been detected through whole exome sequencing (WES) on 3 CLPD-NK patients (all *STAT*-mutation-negative) by Coppe et al. (36). From this analysis, 31 genes harbored somatic mutations including several "cancer genes" i.e., *KRAS*, *PTK2*, *NOTCH2*, *CDC25B*, *HRASLS*, *RAB12*, *PTPRT,* and *LRBA*. More recently, the same authors reported WES data obtained in a larger series of patients indicating that the involvement of JAK/STAT pathway resulted to be not so central as observed in T-LGLL. Otherwise, other pathways and genes were hit by genetic alterations potentially impacting on cell survival, proliferation, chromatin-remodeling, innate immunity, and NK cells activation (41).

Currently, more information is available on ANKL rather than CLPD-NK. Through WES on 14 ANKL patients, Dufva et al. identified alterations in JAK/STAT, RAS/MAPK, and epigenetic modifier genes (42). They found JAK/STAT signaling components frequently altered, with 21% of cases carrying *STAT3*

mutations, showing that some features are shared by ANKL and the relatively indolent LGLL. The same authors demonstrated an overlapping genetic landscape between ANKL and extranodal NK/T cell lymphoma, nasal type (NKTCL) rather than with CLPD-NK, suggesting that ANKL might represent a more advanced form of NKTCL (42).

STAT MUTATION: FOUNDING OR LATE EVENT?

The current pathogenetic hypothesis on LGLL development rests on an initial antigen-triggered oligoclonal LGL expansion that only successively develops into a monoclonal lymphocytosis. In this context, *STAT* mutation seems not to be an inciting but rather an acquired event during the disease, conferring an advantage on the clone development. Indeed, *STAT* mutations are more frequently found in large clones (18). Interestingly, *STAT5b* mutations are more often reported on large monoclonal TCR-Vβ expansions (28), whereas, *STAT3* mutations are also detected in small subclones (18). Kerr et al., evaluating the relationship between *STAT3* mutation and T-cell clone burden, showed that *STAT3* mutation frequency can be lower than the T-cell clone entity thus confirming that the mutation is likely to occur as a secondary event within a pre-expanded immunodominant clone (43). The above authors also observed that *STAT3* mutation may contribute to an autonomous antigen-independent clonal expansion (43).

Many data have been reported demonstrating that *STAT3* mutation does not represent the only factor, itself mandatory, to trigger LGL clonal expansion. *In vitro* inhibition of STAT3 was observed to restore LGL apoptosis independently from *STAT3* mutational status and STAT3 was found activated also in *STAT3* wild type LGLL patients (17). Furthermore, analysis on murine cells transduced with retrovirus showed that *STAT3* mutants (D661V, Y640F) do not provide any cell growth advantage (44). Similarly, a mouse model demonstrated that the expression of *STAT3* mutant alone is not enough to induce LGL leukemia (44), at variance to what had been observed in the mouse model with over-production of IL15 (45). In addition, in a murine bone marrow transplantation model, also Couronne et al. showed that the expression of Y640F mutated STAT3 primarily induces myeloid malignancy rather than LGL disease (46). All these results suggest that additional gene mutations or deregulation due to other signaling molecules or pathways associated with *STAT3* mutations might be involved in LGLL pathogenesis (47).

Several points should be made in terms of the *STAT5b* mutations. *STAT5b* N642H has been indeed identified as an oncogenic driver in innate-like lymphocytes (48), and a mouse model expressing human N642H mutated STAT5b has been described to develop severe CD8+ T cell neoplasia (49). Provided that IL-15 is an upstream factor of STAT5b and that IL-15 transgenic mice develops the aggressive variant of T or NK cell leukemia (50), the IL-15-STAT5 axis might be considered crucial for neoplastic transformation. The requirement of additional cytokine signals on *STAT5b* genetic lesions suggests that the indolent course of CD4+ T-LGLL or Tγδ LGLL carrying these genetic lesions might be due to the lack of one or more concurring events together with *STAT5b* mutations, e.g., cytokine stimulation.

THE CLINICAL IMPACT OF *STAT3* AND *STAT5B* MUTATIONS

Isolated neutropenia represents the clinical hallmark of the disease, observed in 40–60% of patients, with approximately half of them developing severe neutropenia. A significant correlation between the presence of *STAT3* mutations and neutropenia/symptomatic disease has already been highlighted in several studies (13–16, 22, 25). We hypothesized that the mechanism accounting for neutropenia development involves high levels of STAT3 activation (14). More in detail, we recently demonstrated the presence of a STAT3-miR-146b-FasL axis in neutropenic T-LGLL patients, that, once triggered, leads to high production of Fas Ligand, which in turn is responsible of neutrophil apoptosis (51). These data emphasize the role of STAT3 activation in the pathogenesis of LGLL neutropenia, with *STAT3* mutations likely being involved in further boosting this mechanism.

Besides neutropenia, several other clinical features have been described to be more frequent in patients with *STAT3* mutations, including different cytopenias or autoimmune diseases. Interestingly, T-LGLL patients with multiple *STAT3* mutations have been reported to associate with concomitant rheumatoid arthritis (RA) (52). On the contrary, the association with pure red cell aplasia (PRCA) remains a controversial issue (17, 21, 22, 26).

At variance with *STAT3*, *STAT5b* mutation has been reported to have a very different clinical impact. Depending on the immunophenotype of the mutated clone, the presence of *STAT5b* mutations in the same hotspot positions represents a signature of aggressive clinical course with a poor prognosis in aggressive CD8+ T-LGLL patients (28), while it is devoid of negative prognostic significance in CD4+ T-LGLL and Tγδ LGLL patients (15, 29, 53). The issue is quite intriguing since *STAT5b* N642H behaves as a driver mutation in several T-cell lymphomas and in the mice model it is enough to induce a leukemic phenotype (48).

The association between *STATs* mutations and patients' clinical features has been recently confirmed by our data obtained in a large cohort of 205 LGLL patients including all LGLL subtypes, but aggressive T-LGLL and ANKL. We observed that *STAT3* mutations were significantly associated with absolute neutrophils count $<500/mm^3$, hemoglobin level <90 g/L and treatment requirement, while *STAT5b* mutations were found in 15/152 asymptomatic patients. Moreover, by univariate and multivariate analysis, *STAT3* mutated status resulted to be associated with reduced overall survival, firstly demonstrating the adverse impact of *STAT3* mutations in LGLL patients (15).

Considering that a specific therapy is still missing in LGLL and that current immunosuppressive drugs do not provide satisfying responses, the above-mentioned clinical impact of STAT signaling in LGLL makes these molecules attractive new targets for drug development. Several direct STAT inhibitors

interacting with protein domains are available, including Stattic, S3I-201, STA-21 for STAT3 (54) and Pimozide, Stafib2, and Cpd17f for STAT5b (55). However, these compounds induce several off-targets toxicities and severe side-effects that for the time being prevent their use in the clinical setting. To date, no direct STAT3/5b inhibitors of clinical grade are available. However, early-phase clinical trials with drugs targeting STAT3 are ongoing in solid and hematologic malignancies other than LGLL, namely AZD9150, a STAT3 antisense oligonucleotide, and Napabucasin, an inhibitor of gene expression driven by STAT3 (56). In LGLL some compounds against the upstream signaling to STAT have been tested, with preliminary promising results. Tofacitinib citrate, a JAK3-specific inhibitor, showed good response in patients with refractory LGLL associated with RA (30); furthermore, BNZ-1, a multicytokine inhibitor, is currently being tested in LGLL patients in a phase I/II trial (57). Additional studies are needed to confirm these data and the inclusion of *STAT* mutational status in the work-up is suggested to achieve a personalized treatment of LGLL. Consistently, *STAT3* Y640F mutations have been shown to predict response to methotrexate in a small series of patients (7), representing a putative, potential parameter to select the initial best therapy for LGLL patients.

STAT3 AND *STAT5B* MUTATIONS OCCUR IN PHENOTYPICALLY DISTINCT LGLL

The correlation found between *STAT* mutations and LGL immunophenotype has been highlighted, in fact *STAT3* and *STAT5b* mutations are mutually exclusive and preferentially occur in phenotypically distinct leukemic LGLs.

Within Tαβ-LGL disorders, *STAT3* mutations characterize the CD8+ T-LGLL and have never been observed in the CD4+ subset (14, 29). Instead, *STAT5b* mutations have been mainly found in CD4+ TLGLL and also in the rare aggressive form of CD8+ T-LGLL (28), whereas in indolent CD8+ T-LGLL these genetic lesions seem to be rare (14, 29).

Besides this preliminary distinction, a more precise definition of patients harboring *STAT* mutation can be described, considering the differential immunophenotypic combination of the LGL markers, i.e., CD16, CD56, and CD57. In CD8+ T-LGLL, the CD16+/CD56– phenotype, with or without CD57, is strongly linked to patients characterized by the presence of *STAT3* mutation (14). The rare aggressive form of T-LGLL, frequently carrying *STAT5b* mutations, is discretely characterized by the proliferation of CD8+/CD56+/CD16–/CD57– LGLs (28). Interestingly, *STAT5b* mutation is found among CD4+ TLGLL patients whose LGL clone is always CD56+, CD16– (14, 29). Similarly, even though CD16 frequently characterizes Tγδ LGLs, in Tγδ LGLL we observed *STAT3* mutations in CD56– LGLs, whereas *STAT5b* mutations were detected in CD56+ phenotype (53).

Also in CLPD-NK discrete subtypes can be identified by flow analysis. We observed that patients with $CD56^{-/dim}/CD16^{high}/CD57^{-}$ cytotoxic NK cells expansion include a subgroup characterized by a more symptomatic disease and the presence of *STAT3* mutation (39). For ANKL, otherwise, no *STAT* mutation-linked phenotypes have been reported.

Taken together, these data suggest that *STAT3* mutation can occur in CD16+/CD56– LGLs, whereas *STAT5b* mutation may be detectable in CD56+ LGL (**Figure 2**). This link suggests that CD16+/CD56– LGLs and CD56+ LGLs preferentially use *STAT3* or *STAT5b* signaling, respectively, to develop LGL expansion and consequently they are differentially predisposed to be genetically hit. The nature of this relationship remains an open issue that needs to be elucidated. A larger analysis on CLPD-NK and Tγδ LGLL cases is mandatory to get insights also in these less frequent disorders.

CONCLUSIONS

The gain-of-function mutations in *STAT3* and *STAT5b* genes up to now remain the most frequent abnormalities in LGLL, even if additional genetic lesions in STAT-related or cancer genes have been described. The complexity on the genetic background indicates that LGLL can be the result of different mechanisms, emphasizing the heterogeneity of the disease, even if a highly frequent recurrent genetic event has not been demonstrated. Being the most common and specific for this disorder, *STAT3* mutation currently remains the genetic marker suggestive of LGLL, whereas *STAT5b* gene is frequently described mutated also in many other hematologic diseases beyond LGLL.

Molecular analysis of *STAT3* and *STAT5b* is unfortunately not so widely available nowadays, but the evidence that *STAT* mutations have a clinical impact supports the inclusion of this test in the LGLL diagnostic work-up. Moreover, the evaluation of *STAT* mutations is suggested in combination with LGL immunophenotype data to perform an appropriate classification of indolent, symptomatic and aggressive LGLL patients. *STAT3* mutation is indicative of symptomatic disease and reduced patient survival; its evaluation is preferentially suggested for patients affected by CD8 T-LGLL, Tγδ LGLL, and CLPD-NK, particularly when clonal expansion is sustained by CD16+/CD56– LGLs. *STAT5b* mutation has a controversial significance depending on the immunophenotype of the mutated clone. In fact, it is regarded as the signature of an aggressive

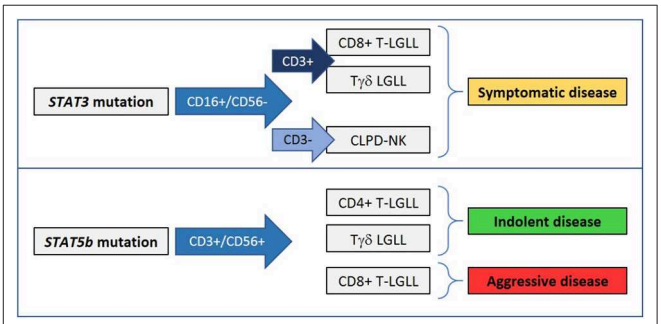

FIGURE 2 | *STAT3* and *STAT5b* mutations are preferentially found in phenotypically distinct LGL disorders and can correlate with different clinical presentations.

clinical course with a poor prognosis in CD8+ TLGLL, characterized by CD56+/CD16–/CD57– LGLs, and aggressive NK cell leukemia, while it is devoid of negative prognostic significance in CD4+ T-LGLL and Tγδ LGLL. Unfortunately, the key factors addressing these two different clinical courses are currently unknown.

STAT3 and *STAT5b* mutations have been included in the 2017 WHO classification of LGLL with the indication that *STAT5b* mutation is associated with a more aggressive disease (12). Now this statement needs to be updated (i) highlighting the correlation between *STAT3* mutation, symptomatic disease and short patient survival and (ii) adding the issue of discovery of *STAT5b* genetic lesions also to indolent CD4+ T-LGLL and Tγδ LGLL. Moreover, in terms of aggressive LGLL harboring *STAT5b* mutation, the current WHO classification recognizes only ANKL, but does not yet recognize the T-related variants as a separate entity. Considering all these findings, the introduction of *STAT* mutation screening as diagnostic tool, together with a correct immunophenotypic analysis, is encouraged for an accurate characterization of LGLL patients.

AUTHOR CONTRIBUTIONS

AT wrote the manuscript. AT, GB, and GC discussed the topics of the manuscript. CV and VG contributed to check the data contained in the manuscript. GS and RZ reviewed, edited, and approved the final version of the manuscript.

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REVIEW
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Next-Generation Sequencing for Clinical Management of Multiple Myeloma: Ready for Prime Time?

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Personalized treatment is an attractive strategy that promises increased efficacy with reduced side effects in cancer. The feasibility of such an approach has been greatly boosted by next-generation sequencing (NGS) techniques, which can return detailed information on the genome and on the transcriptome of each patient's tumor, thus highlighting biomarkers of response or druggable targets that may differ from case to case. However, while the number of cancers sequenced is growing exponentially, much fewer cases are amenable to a molecularly-guided treatment outside of clinical trials to date. In multiple myeloma, genomic analysis shows a variety of gene mutations, aneuploidies, segmental copy-number changes, translocations that are extremely heterogeneous, and more numerous than other hematological malignancies. Currently, in routine clinical practice we employ reduced FISH panels that only capture three high-risk features as part of the R-ISS. On the contrary, recent advances have suggested that extending genomic analysis to the full spectrum of recurrent mutations and structural abnormalities in multiple myeloma may have biological and clinical implications. Furthermore, increased efficacy of novel treatments can now produce deeper responses, and standard methods do not have enough sensitivity to stratify patients in complete biochemical remission. Consequently, NGS techniques have been developed to monitor the size of the clone to a sensitivity of up to a cell in a million after treatment. However, even these techniques are not within reach of standard laboratories. In this review we will recapitulate recent advances in multiple myeloma genomics, with special focus on the ones that may have immediate translational impact. We will analyze the benefits and pitfalls of NGS-based diagnostics, highlighting crucial aspects that will need to be taken into account before this can be implemented in most laboratories. We will make the point that a new era in myeloma diagnostics and minimal residual disease monitoring is close and conventional genetic testing will not be able to return the required information. This will mandate that even in routine practice NGS should soon be adopted owing to a higher informative potential with increasing clinical benefits.

Keywords: multiple myeloma, next generation sequencing, prognosis, personalized medicine, genomics

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MOLECULAR PATHOGENESIS OF MULTIPLE MYELOMA AND RELATED MONOCLONAL GAMMOPATHIES

Multiple myeloma (MM) is a post-germinal center B-cell neoplasm characterized by the accumulation of clonal plasma cells, the production of a monoclonal antibody, and end organ damage (1). MM is preceded by asymptomatic stages of disease in virtually all cases. These are monoclonal gammopathy of unknown significance (MGUS) and smoldering multiple myeloma (SMM). MGUS is a stable condition and progresses at a low rate of 1%/year (2). SMM patients on the contrary have a much higher risk of transforming to MM, but this risk is not constant: it averages 25% per year for the first 2 years, and then declines reaching levels similar to MGUS in patients who did not progress 10 years after diagnosis (3).

The pathogenesis of the disease comes from genomic alterations thought to occur in the germinal center of a secondary follicle of a lymph node. Particularly, cytogenetic changes such as hyperdiploidy or translocations involving recurrent oncogenes and the immunoglobulin heavy chain locus are considered initiating events (4). In some individuals, inherited alleles (i.e., germline polymorphisms) can increase the risk of developing MM but this is considered a rare occurrence and all alleles identified so far have been shown to only confer a small risk (5–14). On the contrary, gene mutations are frequent in newly diagnosed MM (NDMM) and have been particularly characterized since the advent of next generation sequencing (NGS) technologies (15–22). The most commonly involved genes pertain to the MAPK pathway, the NF-kB pathway, the DNA damage response/TP53 pathway. Interestingly, great heterogeneity of the mutational spectrum of NDMM has consistently been reported, such that (i) only few genes are recurrently mutated in a significant fraction of patients, with a high number of genes mutated in <10% of them; (ii) within a single patient, often mutations are only present in a fraction of cells, i.e., they are subclonal (23) or may present in lesions from some anatomical locations but not others (24, 25). Consequently, gene mutations are thought to be late events that contribute to MM heterogeneity and impact disease progression more than its initiation (4). In fact, NGS analysis of sample series has shown variable degrees of spontaneous evolution of genes mutations, cytogenetic lesions and mutational signatures (26–33). This suggests that MM evolves in discrete steps not just clinically but also from a molecular point of view, with the acquisition of subsequent genomic lesions that underlie an increasingly aggressive clinical behavior.

Following examples from other cancers, several efforts have been put in place to use genomics to explain chemoresistance in relapsed disease. Indeed, serial analysis of pre-treatment and relapsed MM samples again showed a tendency toward evolution, where a change in subclonal structure was often observed together with an enrichment of high-risk features (17, 34, 35). Confirming the higher prevalence of high-risk lesions in more aggressive stages, NGS analysis of primary plasma cell leukemia, a rare extramedullary presentation of a clonal plasma cell dyscrasia, showed increased prevalence of *TP53* mutations and del(17p) (36).

Overall, experimental evidence so far suggests that myeloma progression, both spontaneous in asymptomatic stages and at relapse after treatment, is linked to its heterogeneous subclonal composition. Consequently, both the size of the tumor mass and the intrinsic biological features of each subclone must be studied if these biological advances are to be brought to clinical practice to improve prediction of MM evolution.

CURRENT CLINICAL APPROACH TO PROGNOSTICATION IN MONOCLONAL GAMMOPATHIES

Recent advances in NGS technologies have provided us with an unprecedented amount of data on the cell-intrinsic features associated with the natural history of the disease. Despite these advances, diagnostic criteria still segregate MGUS from SMM based on surrogate measures of disease burden (i.e., percent plasma cell bone marrow infiltration and serum levels of the monoclonal protein), and SMM from MM based on the presence of end-organ damage or myeloma-defining events (37).

SMM is a clinical diagnosis that encompasses a wide range of cases, from indolent ones that behave similar to MGUS to aggressive ones that are to progress quickly to MM. Consequently, several risk factors have been proposed to stratify patients based on the risk of progression. Some are based on laboratory values, others on imaging, but only few on intrinsic characteristics of tumor cells: among those, high-risk cytogenetic lesions, gene expression profiling and abnormal immunophenotype (3, 38, 39). However, only rarely such complex techniques are performed in routine diagnosis of SMM. Consequently, the most commonly used risk model for SMM progression relies on % bone marrow plasma cells, levels of the monoclonal protein and free light chains (2, 40–43). Unfortunately, different risk scores show poor overlap (44) and imperfect prediction, which is likely due to the fact that direct measures of the clone size and its intrinsic biological features are not captured by the most widely used approaches.

In NDMM, prognosis has historically been dictated by serum levels of albumin and beta-2 microglobulin within the international staging system (ISS) (45). Only recently the ISS has been complemented by LDH levels and FISH analysis of del(17p), t(4;14), t(14;16) in plasma cells to provide a more accurate measure of risk (R-ISS) (46). Additional studies have shown how the addition of further FISH markers, or the use of SNP arrays can refine prognostication (47–50), but novel prediction scores lack prospective validation and wide applicability so far. Therefore, a lot of variability exists regarding which culture conditions and FISH probes should be used to identify different chromosomal abnormalities (51). This variability stems from the standard practice of each center, but also from national and international guidelines which may slightly differ, and from availability of reimbursement. For example, NCCN guidelines version 2.2020 (https://www.nccn.org/professionals/physician_gls/PDF/

myeloma.pdf) recommend FISH on plasma cells for del(1p), gain (3 copies) or amplification (>3 copies) of chromosome 1q, del(13q), t(4;14), t(11;14), t(14;16), t(14;20), and del(17p) at time of diagnosis. An alternative staging system to the IMWG R-ISS is the Mayo Clinic mSMART 3.0 (www.msmart.org) that stratifies myeloma patients into high or standard risk groups. The former includes del(17p), t(4;14), t(14;16), t(14;20), amp(1q), high risk gene expression profile signature, high plasma cell S-phase, combinations of 2 or more high-risk genetic abnormalities. The latter includes hyperdiploidy, t(11;14) or t(6;14).

UNFULFILLED PROMISES IN MOLECULAR DIAGNOSIS APPLIED TO MYELOMA

NGS has shown how MM genome is characterized by conspicuous heterogeneity and a subclonal structure that gains complexity as the disease evolves. The hypothesis underlying this review is that a precise characterization of this complexity in each patient offers a better possibility to predict, if not prevent, disease evolution and thus improve clinical management. On the contrary, risk scores used in current practice are blind to this complexity, as they rely on clinical and laboratory markers and on a handful of cytogenetic lesions assessed by FISH that are not enough to capture the described complexity and measure MM aggressiveness. NGS thus has the potential to produce a Copernican revolution in how we approach plasma cell dyscrasias in the clinic, i.e., moving from surrogate measures of tumor burden to actual quantification of disease extension coupled with detailed biological analysis of the subclones present in each case.

However, 9 years since the first NGS study in MM has been published (15), clinical practice has been relatively slow in embracing NGS as a diagnostic technique that may complement the standard approach based on morphology, FISH and flow cytometry. Likely, MM intrinsic heterogeneity and the variety of treatment options have hampered the rapid identification of novel prognostic and predictive markers, and there is no consensus so far as to whether, and how, NGS should be used to re-define high-risk disease (52, 53). Furthermore, NGS in MM requires cumbersome sample pre-processing with CD138 cell purification in most cases to obtain meaningful results. As an example, NGS studies in acute myeloid leukemia have gained traction in the clinic in a much quicker way, owing to a lower disease complexity and clearer translational results (54–57).

However, we believe that part of the explanation could also stem from a knowledge gap between routine clinical care and the field of NGS analysis. In fact, NGS can be perceived as a slow, complex, expensive technique that returns results that are hard to interpret and reproduce, and with little clinical value. On the contrary, a targeted NGS panel can inform on gene mutations, aneuploidies, segmental copy-number abnormalities (CNAs), and translocations in a much more comprehensive way than FISH, karyotyping or SNP arrays (58), at a lower cost than a comprehensive FISH panel, with short on-hand processing time and turnaround time and promising clinical correlates. Here, we propose that NGS should be part of the initial diagnostic workup of every NDMM case, at least in tertiary care centers and within clinical trials. This will allow a more precise definition of prognostic and predictive factors that are of clinical significance today. Furthermore, the creation of large NGS data banks that could be mined in the future will allow the quick discovery and validation of novel genomic correlates of prognosis and treatment response that could only become relevant for future treatments.

NEXT GENERATION SEQUENCING

The Technique of Next-Generation Sequencing

In the last decade, the introduction and development of new sequencing technologies opened new biologic scenarios especially in onco-hematologic fields (59). Currently, Illumina (San Diego, CA) and Thermo Fisher (Waltham, MA) are the most used platforms. They are referred to as "next-generation," although effectively they represent a "second generation" of technologies after the irreversible terminator sequencing invented by Sanger. Illumina's integrated NGS instruments use a reversible-terminator based technology. They can read up to 300 bps and importantly, perform paired-end sequencing. This implies that they are able to detect chimeric DNA molecules where the two ends derive from different chromosomes of chromosomal segments, such as in the case of a translocation breakpoint being present in the middle. In fact, many sequencing projects are nowadays aimed at identifing whole-genome translocations and CNAs and not mutations, and to do so a new strategy based on low-coverage, long-insert DNA libraries has been developed to increase the likelihood of identifying chimeric reads at low coverage. This has the potential to represent a new gold standard replacing FISH in the future, since it carries higher accuracy, lower cost and higher throughput. Consequently, Illumina represents the most commercialized NGS platform especially for large genome-wide studies, metagenomics, and gene expression studies (60). Differently, Thermo Fisher sequencing technology, commercialized as Ion Torrent's semiconductor sequencing relies on hydrogen atoms release during DNA polymerization (61). These machines generally provide reads length up to 150–200 bp and are often employed in smaller scale targeted resequencing projects such as those required for diagnostic purposes. As they perform mostly single-end sequencing, their performance in detecting structural variants is weaker.

In the near future, a third generation of sequencing technologies will be widely adopted. The most advanced platforms are provided by Pacific Biosciences and Oxford Nanopores, and they are based on single molecule sequencing without DNA fragmentation, thus producing reads in the thousands of bases. While these are still error-prone machines and not suitable for clinical-grade mutation calls,

they may outperform current technologies for detection of structural variants.

Depending on the input DNA and the processivity of the machines, DNA sequencing can be performed at the level of the whole-genome (WGS), the coding genome (whole-exome, WES) or limited areas of interest (targeted panels). These three sequencing strategies have all been variably adopted to investigate MM heterogeneity. Their principal characteristics are resumed in **Table 1**. Preference between WGS, WES or a targeted panel depends on the type of variants that need to be detected (e.g., mutations vs. aneuploidies vs. structural aberrations) and on the total target footprint. Clearly, smaller footprints allow faster and cheaper analysis, through the possibility of multiplexing more patients into each experiment. Also, IT requirements for downstream analysis are less demanding. For this reason, the choice of the experiment is greatly influenced by the research-diagnostic question, but also by the instrument/technology available and the sample load in each laboratory. Currently, for clinical purposes a targeted panel able to detect mutations, copy number alterations and all the known IGH/IGK/IGL rearrangements represents the most cost-effective solution for risk stratification in MM (21, 35, 58, 62–64). However, this approach is intrinsically limited for research in that it requires prior knowledge of what to look for, and hence it might miss unknown -but relevant- translocations or gene mutations. On the other hand, a WGS approach has the potential to capture the full spectrum of genomic aberrations in MM, but at a much higher cost and time of analysis.

Bio-informatic Analysis

A standard computer cannot process the output of a NGS machine, nor can a clinical scientist analyze it. Rather, a dedicated machine and a bioinformatics data analyst are required to process the raw sequencing output into data of biological and clinical value (**Figure 1**). Bioinformatics is the science that combines knowledge derived from biology, computer science and data analysis, with the aim of understanding and giving a role to data from biological processes of a living organism.

The raw output of an NGS sequencer consists of text files, i.e., strings representing the nucleotide sequences and ASCII-coded values that describe their quality. Based on this quality, each read can be included or discarded, or trimmed of the low-quality bases. Subsequently, the strings of disordered sequences present in the raw files are aligned to the reference genome. One of the most widely used algorithms is the Burrows-Wheeler Aligner (BWA) (65). The alignment output is a binary file containing the mapped reads, which can reach hundreds Gb for a single human genome. The aligned files are then processed to analyze multiple information.

To identify single nucleotide variants and small insertions and deletions (SNVs and INDELs), base calls are compared to the reference genome and often to a matched germline sample from the same patient. The latter is required for larger scale discovery effort such as whole-exome or -genome sequencing, where germline individual variation can lead to the inclusion of a large number of false positive somatic calls. Conversely, small targeted gene panels may be analyzed without a germline control, since the sequencing is only performed to identify recurrent oncogenic somatic variants. Importantly, mutation calls are quantitative, i.e., the frequency of the variant (variant allelic frequency, VAF) is calculated and this is proportional to the number of DNA molecules (and thus to the number of cells) bearing that mutation over the total number of sequenced molecules (cells). This implies that the potential to discover a mutation is proportional to the coverage of the sample, i.e., the average number of sequenced DNA molecules per base of the target region. Coverage can be lower for clonal mutations, that are present in all cells of a tumor and thus in 50 or 100% of reads (for heterozygous and homozygous mutations, respectively). However, coverage must be higher for subclonal mutations, i.e., those present in a limited number of tumor cells, and in samples with low purity where a number of DNA molecules comes from contamination, non-tumor cells. Furthermore, coverage is limited by cost: it can be higher if the footprint of the DNA region is little, and must be

TABLE 1 | Types of tests available for genetic/genomic analysis in MM.

	Target	Cost per sample	Type of variants detected	Number of variants detected	Advantages	Limitations
WGS	Whole genome	∼€1,500 per sample	Mutations (coding and non-coding), indels, aneuploidies, CNAs, structural rearrangements, signatures	Thousands	Comprehensive genomic characterization	Cost, analysis, storage of data, low depth
WES	Coding genome (2%)	∼€500 per sample	Mutations (coding), indels, aneuploidies, CNAs	Hundreds	Lower cost, carries most of clinically useful information, easier analysis	No information on non-coding genome
Targeted	Custom number of genes/regions	Variable	Mutations (coding), indels, aneuploidies, CNAs	Variable	Customizable, lowest cost and complexity of analysis, limited storage required	Not useful for discovery approaches
FISH	Custom number of regions	∼€100 per probe	Deletions, gains, translocations	None	Familiar to most laboratories, short turnaround time	No mutations detected, ideal for a low number of probes

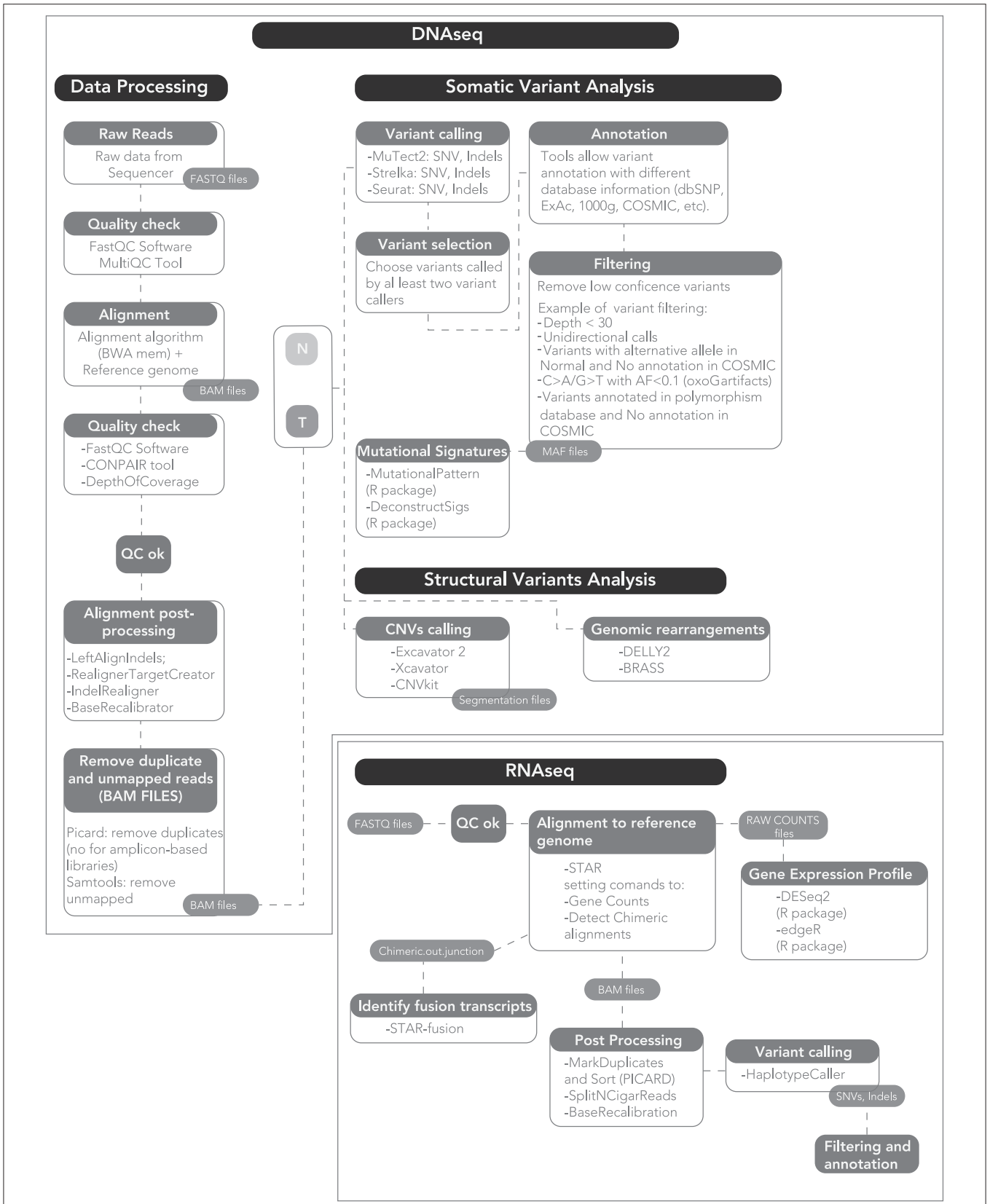

FIGURE 1 | A proposed workflow for comprehensive genomic and transcriptomic analysis.

lower if the whole genome is sequenced. Consequently, there is no set rule to determine the perfect coverage beforehand, and all the factors described above must be considered. As a rule of thumb, a coverage of 30× (i.e., 30 DNA molecules sequenced at each genomic position) is enough to detect clonal mutations and subclonal mutations present in a large fraction of cells, and is typically applied to whole-genome studies. A coverage of 200–500× can allow reliable detection of mutations down to 2–5% of cells and is usually applied to exome studies. A coverage >1000× is usually applied to small targeted panels and can identify variants in <1% of cells, especially with the help of *ad-hoc* algorithms (66). Clearly, this is estimated assuming high tumor purity, which is not always the case.

Different methods to identify variants exist: some are based on allele frequency, counting for each position of the number of normal and alternative alleles, others on probabilistic Bayesian methods, where for each genomic position the probability of observing every possible genotype is returned (67). Importantly, different software can differ in sensitivity and/or specificity, and no gold standard exists to identify variants. However, concordance between different software is usually in excess of 90% for oncogenic variants with high VAF, making calls quite reliable for clinical purposes. However, the concordance can drop to <60% for unknown variants with low VAF, which are nevertheless often discarded in clinic. A new approach that has gained traction is that of using multiple callers to identify variants, and retain only those identified by at least two of them, to increase specificity.

To identify CNAs, NGS offers higher resolution and a more precise identification of breakpoints over conventional arrays, as the depth of coverage in a genomic region is correlated with the total number of DNA molecules sequenced in that region, i.e., its copy number (68). Furthermore, NGS data can be used to evaluate translocations. This analysis is possible when sequencing is performed on a paired-end protocol (i.e., using Illumina machines). Here, opposite ends of the same read that map to distant positions in the same chromosome or different chromosomes are analyzed as they likely highlight a structural rearrangement. Subsequently, single reads spanning the breakpoint can be searched to map the translocation with a base-pair resolution (69).

Another important information that can be evaluated using NGS data is mutational signatures. These are "genomic fingerprints" left around a variant by the biological process that caused it. These are usually processes responsible for DNA duplication and repair, or physical/chemical damage to the DNA. Usually, each process has a preferential activity for a particular nucleotide context, i.e., the base at 5′ and the one at 3′ of the mutation. Combining the six possible types of mutations and the 16 possible contexts, algorithms return the 96-class trinucleotide profile of the mutational spectrum of each sample. This can be further analyzed to extract the mutational signatures (and thus the processes) that contributed to its generation (70).

Last, it must not be forgotten that other types of genetic material can be sequenced. In the case of a cDNA input, NGS can inform on expression levels of genes, expressed mutations, expressed fusion transcripts and splice variants that may have a future in the prognostication in MM. NGS machines can also return information on epigenetic changes related to cancer. Bisulfite-converted DNA can be sequenced to detect methylated cytosines (methyl-seq). Accessible DNA regions can be identified by probing open chromatin with the hyperactive mutant Tn5 Transposase (ATAC-Seq). The activity of transcription factors and the effect of histone modifications can be assessed, along with any other protein-DNA interaction, by sequencing DNA immunoprecipitated with specific antibodies (ChIP-Seq).

CURRENT NGS APPLICATIONS IN MM IN THE CLINIC

At diagnosis, NGS studies are not routinely performed and FISH is still the main approach to molecular characterization of the cancer cells in MM. This carries the intrinsic limitation of investigating only a handful of CNAs and translocations. However, knowing the complexity of the MM genome the amount of information FISH can return is limited, and so could be its prognostic value in comparison with other approaches.

Conversely, the main current application of NGS in the field of MM relies on detection of measurable residual disease MRD through sequencing of the IGH/IGK/IGL loci. This is a very powerful technique, mostly used within clinical trials to date, and mostly through outsourcing the analysis to a commercial service. In a recent meta-analysis, MRD negativity was found to confer an ∼50% relative reduction in the risk of both progression and mortality (71). Historically, molecular MRD has been assessed through a multiplex polymerase chain reaction (PCR) of the IGH locus with consensus primers (72–76) followed by Genescan, heteroduplex analysis, or Sanger sequencing (77). Nevertheless, this approach also amplifies normal B cells resulting in low sensitivity (78, 79). Conversely, an Allele Specific Oligonucleotides (ASO) technique consisting in a real-time PCR with in patient-specific primers and probes has a much higher accuracy and is able to detect up to 1 clonal cell in 100,000 analyzed (80). However, the high rate of somatic hypermutation that occurs in MM cells allows the identification of a molecular marker in only 50–60% of patients. Moreover, detection of the tumor-specific IGH rearrangement often requires cloning of two or more PCR products, resulting in an expensive and labor-intensive procedure. Finally, the ASO qPCR approach does not allow to evaluate the clonal evolution in patients with relapsed MM, thus resulting in false negative results (81). The recent adoption of NGS downstream of consensus primer PCR, resulting in sequencing of all the PCR products, has overcome most of these problems and results in a precise catalog of the IGH, IGK and IGL rearrangements in each case (82). In the diagnostic sample, the tumor-specific rearrangement can usually be easily identified and looked for in the remission sample, always using consensus primers thus increasing the applicability of the technique and allowing a resolution of 1 clonal cell out of a million analyzed cells.

The wide adoption of NGS-mediated MRD measuring with a sensitivity of 10^{-6} is supported by a wealth of clinical data. Recently, Perrot et al. published NGS data from the IFM/DFCI study for young newly diagnosed NDMM patients. In this study, the authors showed that different levels of NGS-MRD cut-off could predict different outcomes in terms of both progression-free (PFS) and overall survival (OS) at both pre- and post-maintenance time points (83). Importantly, the PFS benefit associated with MRD negativity by NGS was similar among the different patient subgroups, thus confirming the theory that MRD is the strongest prognostic marker that overcomes certain adverse risk factors identified at diagnosis (i.e., low-risk cytogenetics and ISS stage II or III), as also reported in other studies (72). The prognostic value of MRD was also independent of previous therapy (transplant vs. no transplant). Moreover, NGS was also explored in other trials for elderly NDMM patients: the ALCYONE and MAIA studies demonstrated that, even if experimental arms (Dara-VMP and Dara-RD, respectively) induced 3- or 4-fold higher rates of MRD negativity compared with control arms (VMP and RD, respectively), the achievement of MRD negativity translated into a significant improvement in PFS independently of previous therapy (84–86). These data are consistent with those of relapsed MM patients enrolled in the CASTOR and POLLUX studies (87–89).

NOVEL INSIGHTS OF CLINICAL VALUE PROMOTED BY NGS IN MULTIPLE MYELOMA

Smoldering Myeloma

The current clinical approach to SMM is watch-and-wait. However, evidence in favor of early treatment is growing, at least for high-risk stages (28, 90, 91). Therefore, improved prognostic scores that could reliably identify high-risk SMM would address a growing clinical need.

Recently, DNA and RNA-based NGS approaches applied to both individual SMM samples and paired SMM-MM cases have shown that these asymptomatic stages carry a globally lower number of mutations than NDMM (28, 29). However, clonal heterogeneity was observed at this stage as well, implying spontaneous evolution of cancer cells through acquisition of new genetic lesions conferring a proliferative/survival advantage. This was particularly true at the level of single-cell RNA, where some cases labeled as MGUS instead revealed plasma cells with a clearly malignant phenotype (33). Interestingly, analysis of serial samples highlighted two patterns of progression: (i) one where cases evolved from minor or entirely new subclones, often without discernible changes of amount of monoclonal protein, and (ii) another where clinical progression was not associated with genomic changes, and was generally quicker (26, 27, 31, 32). Clearly, the former are true asymptomatic cases that need to acquire new lesions to shift their clinical behavior toward an aggressive phenotype, but in the latter case two scenarios are plausible: these are either indolent cases that evolve due to changes in the microenvironment (92, 93), or more likely actual aggressive myelomas that just need more time to accumulate enough tumor burden and/or end-organ damage to meet clinical criteria for progression. Since the advent of NGS, analysis of rearrangements in SMM has been possible at a whole-genome scale. This has been particularly fruitful in the case of MYC rearrangements, which are frequent in MM but hard to study due to promiscuous partners and distant breakpoints. A recent study has clarified how only IGH-MYC rearrangements confer high-risk of SMM progression, mandating that risk scores are updated to reflect this analysis (94). Also complex rearrangements, a newly discovered phenomenon in MM (23), were equally present in SMM albeit at a lower cancer cell fraction (32). Last, a differential timing of activity of mutational processes was observed in SMM: early mutations, likely from pre-cancer initiation stages, arise from the activity of the DNA deaminase AID or from processes associated with cell aging. Late mutations, i.e., the ones arising at the time of disease progression, are more often caused by a cancer-associated mutational process driven by aberrant activity of the APOBEC family of DNA deaminases (32, 95, 96). Therefore, from a genomic point of view, high-risk SMM cases are most similar to NDMM cases and their identification will help stratification of patients (**Table 2**).

Limitations of the described studies are several, ranging from low number of samples, to contamination from normal cells in some cases, to an inevitable bias toward higher-risk SMM cases. However, data are promising enough to believe that NGS will in the future unravel the actual determinants of disease progression in SMM and that will have a profound impact on clinical management of asymptomatic patients.

Newly Diagnosed Myeloma

At diagnosis, MM is staged in three risk groups based on the R-ISS that relies on surrogates of disease burden (albumin, beta-2 microglobulin, LDH) and FISH for three high-risk cytogenetic features (t4;14), t(14;16), del(17p) (46). Clearly, the extended genotyping ability provided by NGS holds the promise of further refining the prediction of such risk (19, 21, 95, 98), as well as that of identifying novel predictive markers that may guide treatment (99–101). However, the prognostic information at diagnosis in MM has historically been only relevant for the patient in terms of management of expectations, as no risk-adapted treatment in myeloma is available. Instead, the landscape is rapidly changing, and four main aspects are to take into account when thinking of prognostication and treatment of myeloma in the coming years: (i) not all high-risk prognostic factors are captured by the R-ISS (52, 53); (ii) novel treatments may significantly change the catalog

TABLE 2 | Papers showing a prognostic value of extended genotyping in smoldering myeloma.

Study	Method of detection	What detected
Bolli et al. (32)	WGS	APOBEC signature
Maura et al. (23)	WGS	Complex rearrangements
Misund et al. (94)	WGS, targeted	IGH-MYC translocations
Boyle et al. (97) (ASH abstract)	WES, targeted	Mutational burden

of high-risk features; (iii) treatment may become risk-adapted soon; (iv) predictors of response, even if devoid of prognostic value, may soon enter clinical practice.

Regarding the first point, the high-throughput genotyping possibilities offered by NGS, coupled with the availability of large datasets amenable to analysis, have highlighted several risk factors that go beyond the ones described above. Perhaps the most cited ones are those included in the definition of "double hit" MM, where features such as amp(1q) in the context of R-ISS stage III and bi-allelic inactivation of *TP53* by means of mutations of one allele and deletion of the other confer poor prognosis independently of the R-ISS. Importantly, the prognostic value of CNAs in chr1q seems limited to amplification of 4 or more copies of the chromosome, a quantitative result that NGS can capture (98). Other markers have a less clear impact. Among those are del(1p) and del(12p)(48) and a rare state of hypodiploidy/hyperaploidy associated with del(17p) (102–104), which is evident by karyotyping and where NGS could be particularly informative. Furthermore, the recent discovery of the poor prognostic value of immunoglobulin lambda translocations and their lack of response to IMiDs (105) highlights once more the value of an "unbiased," whole-genome approach in discovery of prognostic markers. In contrast, single gene mutations seem to have very little prognostic value in most cases (17, 21). The one exception is the Myeloma XI UK trial, where patients were treated with IMiDs in first line. In this context, *EGR1* and *IRF4* mutations conferred good prognosis, and *ZFHX4* a bad one (19). However, the study of the whole mutational spectrum of NDMM genomes allowed to draw some correlations between hypermutated samples and worse prognosis (17, 22). This concept can be extended to the analysis of cytogenetic lesions, where several papers have highlighted that prognosis is inversely proportional to their number, often independently of their type (21, 47, 49). Lastly, initial reports on the analysis of mutational signatures to prognosis have highlighted that cases with high contribution from APOBEC have worse prognosis independently from the number of mutations and the cytogenetic subgroup (95, 106). Altogether, data collected in the last years are pointing at a much larger array of lesions that need analyzing to accurately prognosticate NDMM, as it looks like survival is influenced by an increasing genomic complexity more than the presence/absence of a handful of genetic lesions. Unsurprisingly, novel risk scores are emerging that take into account a larger number of lesions to improve prognostication in NDMM (50) (**Table 3**).

TABLE 3 | Papers showing a prognostic value of extended genotyping in newly diagnosed myeloma.

Study	Method of detection	What detected
Palumbo et al. (46)	FISH	t(4:14), t(14;16), del(17p)
Carballo-Zarate et al. (49)	Karyotype, FISH (HDMM patients only)	del(1p), amp(1q), t(11;14), del(13q), del(17p)
Bolli et al. (21)	Targeted	197 different events (mutations, CNAs, translocations)
Maura et al. (95)	WES	Mutational signatures
Walker et al. (22)	WES	Any driver gene mutation
Walker et al. (98)	WES	TP53 mutations, amp(1q), t(4:14), t(14;16), del(17p)
Perrot et al. (50)	FISH, Cytoscan HD arrays	Trisomy 5, Trisomy 21, t(4;14), amp(1q), del(1p32), del(17p)
Barwick et al. (105)	WGS	IGL translocations

In addition to the well-established role of genomic lesions in the onset and development of MM, deregulated epigenetic mechanisms are emerging as important in MM pathogenesis and prognosis. In the past decade, several studies have suggested that epigenetic mechanisms via DNA methylation, histone modifications and non-coding RNA expression are important contributing factors in MM. Their relevance ranges from disease initiation, progression, clonal heterogeneity and response to treatment. All of these post-translational modifications (PTMs) can be tested by next-generation sequencing, focusing on the status of a single gene or small group of genes, potentially revealing their impact on patients' prognosis. For example, in MM global DNA hypomethylation correlates with disease progression (107) and poor prognosis (108). Moreover, DNA methylation has been shown to influence the expression of microRNA genes with tumor suppressor functions (109–111). Deregulation of miRNAs expression and function has been suggested to have a clear impact on tumor initiation, progression and metastasis in cancer including MM (112–114). Global analysis of miRNA expression in MM has also revealed a clinical relevance as the analysis could correlate miRNA expression to disease progression, molecular subtype, survival and response to treatment (115–120). More recently, several whole genome sequencing and gene expression studies have underpinned that histone PTMs can model chromatin structure driving complex regulatory networks (121). However, epigenetic mechanisms are far from reaching enough evidence to be proposed as clinical-grade prognostic markers and further work and technological advances are needed before this can happen.

Whole transcriptome analysis, both by microarrays or RNAseq, can also be used to identify gene expression signatures with prognostic value. The University of Arkansas for Medical Sciences (UAMS) group has proposed some years ago the GEP70 test as a significant predictor of outcome, independent of clinico-pathologic and genetic features (122). More recently, the SKY92 signature has been validated and combined with the International Staging System (ISS) to identify patients with different risk disease with high sensitivity (123). Despite extensive validation and convincing results though, gene-expression based prognostic scores have not gained widespread adoption. Problems are a lack of consensus over which signature should be used, and a laborious and non-standardized sample processing and data analysis.

Therefore, genetic and genomic markers are by far the prognostic markers in MM that are closer to clinical adoption. However, high-risk features are also necessarily defined relative to the treatments available. The one exception is del(17p), that is universally confirmed across age groups and treatment types.

Examples of less stable features include the t(4;14), which seems to respond well to first-line bortezomib (99), similar to cases with deletion of the TRAF3 gene (124). On the contrary, the negative prognostic effect of *ZFHX4* mutations seems limited to patients receiving IMiDs as first line, as discussed above. Likely, with an increasing array of anti-myeloma agents, this list is going to expand realizing the much-valued paradigm of precision medicine through the identification of further correlates of drug response. Again, this will mandate that extended genotyping is performed at diagnosis for every patient.

Finally, the treatment landscape of NDMM is rapidly changing thanks to the introduction of novel agents and combinations. Risk-adapted treatment is already proposed by some groups, e.g., with respect to performing or not autologous stem cell transplantation (ASCT) in first line for standard-risk patients (125, 126). Conversely, tandem ASCT has shown improved survival in patients with high-risk features and is widely used in Europe in this setting (127). Last, the introduction of minimal residual disease (MRD) monitoring in people achieving deep responses also carries big promises. MRD-negative status seems to predict longer-term survival regardless of the treatment administered and of the risk at diagnosis (83), so that in future clinical trials it may become a new standard endpoint. In fact, many upcoming clinical trials are designed with different treatment arms based on the risk score at diagnosis and on the achievement of MRD-negative status, so that the future may bring innovative strategies to personalize treatment in MM.

Relapsed and Refractory Myeloma

Much less is known about the genomics of relapsed-refractory myeloma. Initial studies suggest that cases retain a significant heterogeneity, with subclones showing expansion or reduction based on the type of treatment, increased number of mutations, copy-number abnormalities, complex rearrangements and contribution from novel mutational signatures (17, 23, 34, 35, 96). Targeted sequencing studies have highlighted increasing prevalence of mutations conferring resistance to IMiDs (particularly in *CRBN, IKZF1, IKZF3*) and PIs (*PSMB5, PSMB8, PSMB9, PSMD1, and PSMG2*) (62, 128). However, mutated cases are still a great minority and mutations are often subclonal, suggesting that while functionally relevant, the clinical impact of these mutations and their utility to guide further treatment will need validation. To little surprise, single-agent targeting of actionable gene mutations has revealed unsatisfactory in MM so far (24, 129, 130).

On the contrary, analysis of cases refractory to both Pis and IMiDs has highlighted a higher prevalence of high-risk cytogenetic features such as amp(1q) and del(17p) that may explain chemoresistance much more readily (131). The evidence that new mutations and cytogenetic lesions can be acquired at relapse suggests the utility to repeat genotyping at this stage. However, future studies will also be required to assess whether the predictive and/or prognostic value of genomic alterations described in NDMM is conserved in advanced stages.

FUTURE CLINICAL APPLICATIONS OF NGS IN THE CLINIC

The recent advances described above suggest that, in the near future, routine management of MM will require such a vast array of genetic findings that an NGS platform would be perfectly suited to address this need (**Figure 2**). NGS has been already shown to perform as well as or even outperform FISH for structural changes (58, 63, 64). However, cost, turnaround time and regulatory aspects also need to be taken into account. Given the growing number of FISH probes required for a comprehensive characterization of MM and the decreasing cost of sequencing, there is little doubt that soon targeted NGS panels, or even WGS will become cheaper than running, for example, 8 FISH probes as per NCCN guidelines. Turnaround time of FISH can be as short as 24 h. NGS requires slightly more time than this, in the order of 2–3 working days as a minimum. However, treatment of MM is rarely an emergency, and even most acute cases of cord compression or renal failure can be managed with a short course of steroids while waiting for test results. Regulatory aspects are more difficult to discuss as they are also crucially variable from country to country, or even in different regions of the same country. But given the above considerations and the added value of NGS sequencing over FISH, there is little doubt that authorities will allow reimbursement of a cheaper, solid and more comprehensive test than FISH.

Prognosis in Asymptomatic Stages

In SMM, NGS approaches could capture complex genomic information that may be relevant to stratify risk of progression. Data such as the total mutational burden, the presence of complex rearrangements and IGH-MYC rearrangements in particular, the contribution of APOBEC mutational signature would need a comprehensive assessment through whole-genome sequencing. Techniques such as mate pair sequencing (63) may be particularly well-suited to identify IGH-MYC translocations and complex rearrangements due to their erratic breakpoints and promiscuous partners, features that limit FISH accuracy in detecting these events (132). While costs may limit this approach, a targeted panel of gene mutations, recurrent translocations, and CNAs could still improve our prognostication abilities over current biochemical and imaging techniques.

Prognosis at Diagnosis and After Induction

In NDMM, NGS analysis will inform on current higher risk cytogenetic markers as per IMWG recommendations. But more importantly, genome-wide copy-number analysis will inform on genome-wide CNAs, aneuploidies and translocations that may change the prognosis of the patient. The simultaneous analysis of gene mutations will provide an added value for prognostication, particularly for *TP53* mutations. Clearly, the quantitative nature of NGS data will also provide an estimate of the number of cells affected by each lesion, which has clinical correlates. Also, a comprehensive mutational analysis of each case will highlight cases that are hypermutated and those with an increased contribution from APOBEC, again markers of high-risk disease.

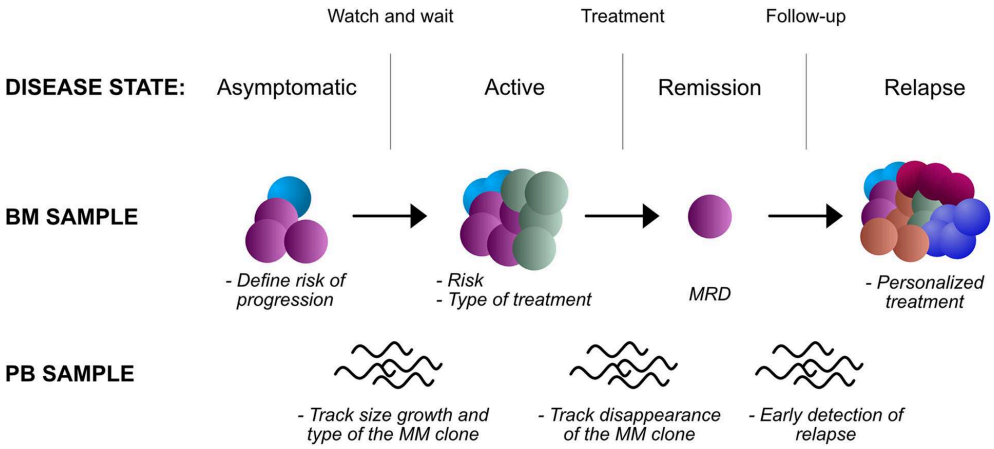

FIGURE 2 | Potential applications of NGS in the clinic, in different disease states and biological samples. BM, bone marrow; PB, peripheral blood; MRD, minimal residual disease.

After induction treatment, NGS can be used to assess MRD as described above. This is probably the most mature application of NGS, and the one that is to see adoption in routine clinical practice first. Hurdles that will need overcoming are the standardization of a protocol to ensure inter-laboratory comparability, access to sequencing facilities and high costs. However, even the more standardized and culturally more accessible use of flow cytometry, despite providing results of similar prognostic value (133), has not gained universal adoption. In the near future, the MRD status may not only be used to inform the patient on his prognosis, but also to guide post-remission treatment -which drugs and for how long- and the need for an autologous stem cell transplant.

TABLE 4 | Genomic and trasncriptomic correlates of drug response in RRMM.

Study	Method of detection	What detected
Andrulis et al. (129)	Immunohistochemistry	*BRAF*^{V600E}
Heuck et al. (130)	Targeted, gene expression profile	KRAS, NRAS, BRAF mutations
Kortüm et al. (62)	Targeted	*CRBN* and CRBN pathway genes
Barrio et al. (128)	Targeted, WES	Proteasomal subunit genes
Kumar et al. (100), Matulis et al. (134), Neri et al. (135) (ASH abstract)	FISH, functional studies, gene expression arrays, single cell RNAseq	t(11;14), BCL2/MCL1, and BCL2/BCL-XL RNA ratio, mitochondrial priming

Prognosis at Relapse: Time for Personalized Care?

In RRMM, further BM sampling and genomic analysis may provide improved prognostication and correlates of drug response (**Table 4**). Examples impacting current clinical practice include the use of second-line ixazomib, that in Italy is only reimbursed upon demonstration of a high-risk cytogenetic status that may be absent at diagnosis. More broadly, recent results suggest that a "targeted treatment" could be closer than expected in the RRMM setting. The most promising results come from venetoclax, a novel inhibitor of the anti-apoptotic BCL-2 protein, which carry single agent activity in RRMM (100) and has shown impressive survival data in combination with bortezomib and dexamethasone. Intriguingly, these advantages are evident in the t(11;14) cytogenetic subgroup (136), again mandating detailed genotyping of the disease. However, BCL2/MCL1 and BCL2/BCL-XL RNA ratio appear to be equally good predictive markers for venetoclax response (134, 137), while *BCL2* mutations and *MCL1* amplification predict resistance (135). This suggests that RNAseq analysis along with DNA sequencing could improve stratification of patients. Use of BRAF inhibitors in *BRAF*^{V600E} mutated cases is an approach that failed to fulfill initial promises due to the quick onset of resistance. However, selection of cases where the mutation is clonal, and the combined use of MEK inhibitors could hold promise for the future (24, 130). A more speculative example would be represented by the inhibition of EZH2 in UTX-deleted cases (138). Other mutations could carry a negative predictive value: hotspot *CRBN* mutations suggesting resistance to lenalidomide (62); proteasomal subunit mutations predicting resistance to bortezomib (128); *XPO1* mutations predicting resistance to selinexor (139). Interestingly, most such lesions are often subclonal, and single cell sequencing techniques are starting to uncover an unexpectedly complex spectrum of phenotypes within the myeloma bulk (135). However, despite their potential, such approaches are still far from a possible clinical application.

Finally, novel studies are aimed at incorporating a precision medicine approach in MM. The MyDRUG study (clinicaltrials.gov ID NCT03732703) has six arms where a backbone of ixazomib, pomalidomide, and dexamethasone is used in conjunction with a targeted agent aimed at mutations of any of the following genes: *CDKN2C, FGFR3, KRAS, NRAS,* *BRAF*^{V600E}*, IDH2,* or t(11,14). Clearly, inclusion in the study mandates comprehensive NGS analysis.

Mini-Invasive Approaches for Genotyping and Prognostication

The analysis described above rely on cellular DNA from BM CD138$^+$ cells. However, a major breakthrough may be represented by mini-invasive approaches based on circulating tumor cells (CTCs) and circulating cell-free DNA (cfDNA). At diagnosis, these approaches have proven reliable in describing the main clonal gene lesions and aneuploidies in the majority of NDMM patients, where enough tumor cfDNA and/or CTCs are present (140–142). However, a fascinating possibility is that of applying these "liquid biopsy" approaches to add further accuracy to progression free survival (PFS) prediction by defining MRD negativity. In fact, while cellular (e.g., flow cytometry) and molecular (NGS of IgH rearrangements) methods for MRD detection are very sensitive, they are restricted to a single-site BM biopsy, which is in contrast to the patchy and heterogeneous pattern of bone marrow infiltration observed in MM. This may lead to some degree of uncertainty in MRD-negative results, where the disease can still be present away from the bone marrow sampling site. The proof of concept of this caveat is illustrated by the presence of MRD negative patients that still display a monoclonal protein at serum protein electrophoresis (82), and by the poorer survival of a small fraction of MRD-negative, PET-positive patients (143). Some research groups have explored the feasibility of cfDNA analysis to monitor IGH rearrangements by adopting different NGS MRD approaches (144–146). However, the number of CTCs and genome equivalents in cfDNA is so low in MRD settings that these techniques, although feasible, still lack several logarithms of sensitivity before they can reach 10^{-6} and be proposed as standard approaches. Likely, a technical advance is required before the number of tumor genomes sequenced is maximized and this technique provides increased sensitivity.

Another way to longitudinally monitor MM patients and the competitive emergence of subclones through cfDNA is the evaluation of the allelic fraction of known mutations. Some studies have reported the possibility to monitor a single mutation (such as $BRAF^{V600E}$, $NRAS^{Q61K}$) by serial sampling of ctDNA (147, 148). This represents a valid approach that takes into account the spatial heterogeneity of MM, and its usefulness would be maximal if the mutation in study is druggable or predicts response to treatment. Another study employed a targeted panel to characterize paired BM and PB samples before treatment. Patients with a higher number of mutations or a higher mutational fractional abundance in PB had significantly shorter overall survival (OS). Moreover, a decrease in ctDNA levels at day 5 of cycle 1 of treatment (C1D5) correlated with superior progression-free survival (PFS) ($p = 0.017$) (149).

Interestingly, ctDNA can be also used to track disease load and clonal evolution of MM by low pass WGS (142). This method allows the identification of copy number alterations even when the tumor load is relatively low. In a pilot study, the potential of cfDNA as a longitudinal marker for disease progression and therapy response has been explored. A patient was monitored before and after therapy both in BM and ctDNA, and efficacy of therapy was evident by decreasing levels of serum free light-chains (sFLC) and concordant trajectory of tumor fraction in cfDNA. The cfDNA copy number profiles on day 0 and day 19 (with no change in management) were concordant. Tumor fraction became undetectable with response to treatment (days 41, 69). However, with relapse extensive clonal evolution occurred (day 224, after relapse) as drug resistance developed. Importantly, the copy number profile of cfDNA and BM on day 224 were concordant. These promising evidences need to be further confirmed by additional studies, and probably pave the way to the use of ctDNA for disease monitoring in the near future.

Once validated, the usefulness of ctDNA, both by IGH rearrangements and/or mutations and/or tumor fraction, together with indirect immunobiochemical markers (e.g., monoclonal protein) and imaging techniques (such as PET-CT or WB-MRI) could possibly help to re-define more precisely the minimal residual disease in MM. Even more interesting, these approaches could be applied to the setting of SMM monitoring, where increase rather than decrease of tumor cfDNA would be observed in progressive cases: this could in theory be less technically challenging and provide earlier detection of symptomatic evolution.

AUTHOR CONTRIBUTIONS

NB and CT conceived the idea, reviewed literature, and wrote the paper. EG, BZ, MM, and SO reviewed literature and wrote the paper.

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RNY4 in Circulating Exosomes of Patients With Pediatric Anaplastic Large Cell Lymphoma: An Active Player?

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Emerging evidence indicates that extracellular vesicles, particularly exosomes, play a role in several biological processes and actively contribute to cancer development and progression, by carrying and delivering proteins, transcripts and small RNAs (sRNAs). There is high interest in studying exosomes of cancer patients both to develop non-invasive liquid biopsy tests for risk stratification and to elucidate their possible involvement in disease mechanisms. We profiled by RNA-seq the sRNA content of circulating exosomes of 20 pediatric patients with Anaplastic Large Cell Lymphoma (ALCL) and five healthy controls. Our analysis disclosed that non-miRNA derived sRNAs constitute the prominent fraction of sRNA loaded in exosomes and identified 180 sRNAs significantly more abundant in exosomes of ALCL patients compared to controls. YRNA fragments, accounting for most of exosomal content and being significantly increased in ALCL patients, were prioritized for further investigation by qRT-PCR. Quantification of *RNY4* fragments and full-length sequences disclosed that the latter are massively loaded into exosomes of ALCL patients with more advanced and aggressive disease. These results are discussed in light of recent findings on the role of *RNY4* in the modulation of tumor microenvironment.

Keywords: ALCL, liquidbiopsy, exosomes, YRNA, RNA-seq, small RNA

INTRODUCTION

Extracellular vesicles (EVs) are cell-derived membrane particles secreted from many cell types and circulating in body fluids, including plasma. Among different classes of EVs of different size and intracellular origin, exosomes are 40–150 nm endosome-derived EV originating from the inward budding of the limiting membrane of multivesicular bodies (1). In this process, exosomes are packed with proteins, lipids, DNAs, messenger RNAs (mRNAs) and non-coding RNAs, which can be transferred to recipient cells, and function both as paracrine and endocrine factors (2).

A large body of evidence collected in the last years proved the functional involvement of exosomes in cancer progression and spreading, induction of angiogenesis, as well as in

chemoresistance and immune response evasion during tumor development (3). In this scenario, defining the peculiarities of exosomal cargo in cancer patients is a hot topic in biomedical research. The characterization of small non-coding RNAs (sRNAs) in plasmatic exosomes of cancer patients attracted interest for the identification of non-invasive disease biomarkers and, notably, in consideration of sRNA regulatory functions and their direct involvement in cancer mechanisms (4, 5).

Most functional studies on circulating sRNAs carried by tumor-derived exosomes were focused on microRNAs (miRNAs) because of their well-characterized regulatory roles in key signaling axes: exosome-delivered miRNAs have been shown to promote epithelial-mesenchymal transition (6, 7), induce angiogenesis and increase vascular leakage (8–10), prepare pre-metastatic niches to promote metastasis (11, 12) or induce tumor resistance to immune responses (13, 14).

Non-Hodgkin lymphoma (NHL) is a heterogeneous group of lymphoid malignancies and the fourth most common malignancy across the pediatric age spectrum. Considerable progress has been achieved in developing curative therapy for pediatric NHL, with an overall survival rate now exceeding 80% (15). There are three major categories of NHL: mature B-cell neoplasms, Lymphoblastic Lymphoma, and Anaplastic Large Cell Lymphoma (ALCL). Other NHL subtypes, including peripheral T-cell lymphomas, follicular lymphomas, and rare entities, represent <3% of the cases (16).

ALCL accounts for 10–15% of pediatric and adolescent NHL. Differently from ALCL in adults, ALCL in children is nearly universally ALK-positive and, in almost all of the cases, it is characterized by the *t*(2;5)(p23;q35) translocation, which leads to the constitutive expression of the NPM-ALK fusion protein (17). Although current treatment strategies achieve an event-free survival (EFS) of ∼75% after 5 years, about 30% of the patients are resistant to therapy or experience a relapse (18). In this clinical context, new disease biomarkers are needed to enable the early identification of high-risk patients and a better tailoring of treatment. In ALCL, the identification of new active players in lymphomagenesis and in cancer cells dissemination mechanisms would have high potential for the design of innovative therapeutic interventions.

Currently, only a few, mainly descriptive, studies reported data regarding the sRNA cargo of lymphoma-derived exosomes and EVs. Moreover, only Diffuse Large B-cell Lymphoma (DLBCL), which is the most frequent histological subtype presenting in adults (19, 20), was investigated.

Studies aiming at the identification of clinically relevant sRNAs in plasmatic exosomes/EVs from lymphoma patients focused on miRNAs (21). The first evidence on EV miRNAs as a molecular diagnostic tool for disease monitoring in Hodgkin lymphoma (HL) patients was reported by Eijndhoven et al. (22). Specifically, lymphoma-associated miR-21-5p, miR-127-3p, miR-24-3p, let-7a-5p, and miR-155-5p were significantly increased in plasmatic EV from HL patients compared to healthy donors (HD). Exosome-derived miRNAs were also proposed as predictive biomarkers of chemotherapy resistance in DLBCL, where increased levels of miR-99a-5p and miR-125b-5p in patient plasmatic exosomes were associated with reduced progression-free survival (23). Data on exosomal sRNAs in pediatric lymphomas setting are currently missing.

Further, our appreciation of the small transcriptome complexity largely increased in the last years. Alternative processing of miRNA precursors (24–26) and housekeeping non-coding RNAs can generate miRNA-like sRNAs that can be functional and play roles in malignancies (27, 28). It's worth noting that several RNA-seq studies on exosomes derived from both tumoral and non-tumoral cell lines revealed that, differently from secreting cells, miRNAs constitute a minor percentage of EV-enclosed RNA. Besides protein coding mRNA, the EV fractions contain vault RNAs, YRNAs, small nuclear and nucleolar RNAs (snRNAs and snoRNAs), transfer RNAs (tRNA), as well as fragments deriving from long non-coding RNAs and transcribed pseudogenes (29–31).

In this perspective article, we present original data about the characterization of exosomal sRNAs in pediatric ALCL aiming attention on non-miRNA derived sRNAs, and discuss them in the frame of current literature, providing an original viewpoint of the possible translational relevance of these findings.

RNY4 FRAGMENT ENRICHMENT IN CIRCULATING EXOSOMES OF ALCL PATIENTS DISCOVERED BY RNA-seq

To characterize the exosomal load of sRNAs in ALCL patients and disclose differences with healthy donors, sRNAs were examined by small RNA-seq of exosomes from 20 ALK-positive ALCL patients and five HD plasma samples, and from supernatant of five ALCL cell lines (Karpas299, SUDHL1, and SUP-M2, ALK-positive; FE-PD and MAC2A, ALK-negative). For patients, paired biopsy samples, all positive for the NPM-ALK fusion, were also sequenced. Exosomal RNA was extracted by using exoRNeasy Maxi/Midi kit (Qiagen) and assessed for proper amount and quality by Agilent 2100 Bioanalyzer (Agilent Technologies). RNA-seq libraries were prepared with NEBNext Multiplex Small RNA Library Prep Kit for Illumina (New England Biolabs), as previously reported (32), and sequenced on an Illumina HiSeq 4000 platform with single-end reads and average depth of 15 and 30 M for exosomal and biopsy samples, respectively.

After a preprocessing phase for adapter trimming and selection of high-quality reads (Qphred $\geq$ 30), data underwent analysis by miR&moRe software (24, 25). Briefly, miR&moRe maps filtered reads to genome assembly and the known hairpins sequences from miRBase extended in either directions by additional 30 bp and allows detection and prediction of miRNA hairpins and of the corresponding mature forms, as in Gaffo et al. (33). MiR&moRe allowed identification and quantification of 1,194 and 523 miRNA-derived sRNA species in biopsies and exosomal samples, including miRNAs and microRNA-offset RNAs derived from annotated and newly predicted miRNA precursors.

We observed that in biopsies ∼59% of the reads derived from miRNAs, whereas the large majority of the reads from exosomal samples did not align to known nor predicted miRNA precursors,

concordantly in exosomes isolated from ALCL patients (2.5%), cell lines (2.6%), and healthy donors (5.3%) (**Figure 1A**). A similarly large fraction of non-miRNA sRNAs in EVs was described by other studies on lymphoma, melanoma, breast cancer and immune cells (19, 20, 29–31).

The non-miRNA sRNAs fraction in exosomes of ALCL patients was further characterized. First, reads not aligned to known or predicted miRNA precursors were mapped with Bowtie v1.1.2 (34) to the reference genome, allowing no mismatches and up to 15 multiple alignments. The alignments were then analyzed with *derfinder* software tool (35) to identify expressed RNAs and the corresponding genomic regions. Among the 9,181 regions supported by at least 10 reads we selected those of 13–50 contiguous bases, consistently with the size of sequencing library fragments. After expression normalization [DESeq2 (36)], 1,007 most abundant (top 5% of expression) putative non-miRNA-derived sRNAs were considered for further characterization. Principal component analysis of expression profiles of these 1,007 sRNAs clearly distinguished tissue from exosome samples, separating as well as exosomes from healthy donors, patients and cell lines (**Figure 1B**).

Next, 180 sRNAs were identified with significantly different abundance between exosomes of ALCL patients and HD (DESeq2 p.adj < 0.001). The heatmap in **Figure 1C** shows the expression of the nine sRNAs with highest abundance and increase in ALCL exosomes. Apparently, the sRNA present in greater supply in ALCL exosomes was defined by reads aligned to the *RNY4* gene, and to highly similar pseudogenes (*RNY4P7*, *RNY4P10*, and *RNY4P20*). Precisely, a fragment corresponding to the first 32 bases on the 5′ end of *RNY4* (*RNY4*-5′F) (**Figure 1D**) accounted for at least 80% of the non-miRNA sRNA expression in exosomes, whereas it was less abundant in exosomes derived from the cell line supernatant, isolated with the same protocol (**Figure 1E**). Remarkably, *RNY4*-5′F was five times and significantly more abundant in ALCL than in HD exosomes (p.adj $= 0.0003$; **Figure 1F**). The expression of *RNY4*-5′F was assessed by quantitative real-time PCR (qRT-PCR; Custom TaqMan Small RNA assay designed on the 32 bases of the *RNY4* sequence; ThermoFisher Scientific, Life Technologies) in 25 independent samples (12 ALCL and 13 HD). No difference was observed comparing exosomes from ALCL and HD (**Figure 1G**) not confirming RNA-seq result (**Figure 1F**).

YRNAs were first discovered in 1981 as 83–112 nt RNA components of circulating ribonucleoproteins, complexed to Ro60 and La autoantigens, in serum of patients with autoimmune diseases (37, 38). Evolutionary conserved in vertebrates (39), YRNAs fold in characteristic stem-loop secondary structures, with lower and upper stem loop sequences being the most conserved (40). Four different human YRNAs (*RNY1*, *RNY3*, *RNY4*, and *RNY5*) are transcribed in the nucleus by RNA polymerase III from genes clustered together at a single locus on chromosome 7q36 (41). Intracellularly, binding of the lower YRNA stem to Ro60 was shown to be involved in the maintenance of RNA stability and in cellular response to stress (42), whereas the upper stem was proven to be essential for the initiation of chromosomal DNA replication (43). In addition, YRNAs were also linked to alternative splicing and regulation of the translation of specific RNAs, since most YRNA-associated proteins are implicated in these processes (44).

In recent years, YRNAs and YRNA fragments derived from site-specific cleavage by RNase L were reported to be enriched in different types of EV compared to secreting cancer cells (19, 31, 45–47). Noteworthy, RNA fragments corresponding to the 5′ region of the *RNY4,* almost exactly corresponding to the *RNY4*-5′F detected in the present study, were shown to be the most abundant sRNA species in plasma samples from HD (30, 48–50) and melanoma patients (51), as well as in breast cancer patients' exosomes and plasma (30, 49), and plasmatic exosomes from non-small cell lung cancer (NSCLC) and chronic lymphocytic leukemia (CLL) patients (52, 53).

Since YRNA fragments derive from conserved ends of the YRNA hairpin, it was initially hypothesized that YRNAs could "conceal miRNAs" and be processed in miRNA-sized YRNA fragments that could function as miRNAs (54). However, this hypothesis was not supported by later studies. Since YRNA fragment biogenesis resulted to be Dicer-independent, they were found in complexes different from those associated with microRNAs and they did not co-immunoprecipitate with Ago2 (55). Moreover, they did not regulate targets tested by Thomson and colleagues in a miRNA-like manner (56).

Recently, since *RNY5* fragments administration to human primary fibroblasts was shown to induce cell death (46) a role for *RNY3* in enhancing "cleavage and polyadenylation specificity factor" (CPSF) recruitment to histone locus bodies has been proposed (57), thus associating YRNA fragments to functional activities different from those typical of miRNAs.

GENUINE miRNA-LIKE *RNY4* FRAGMENTS OR FULL-LENGTH *RNY4*?

The increased levels of *RNY4*-derived fragments or full-length transcripts circulating in plasma or exosomes of cancer patients compared to HD (30, 49, 53) triggered interest in the potential use of *RNY4* as a cancer biomarker. However, whether *RNY4*-derived fragments detected by small RNA-seq are genuine fragments or reflect the presence of the full-length *RNY4* is still a matter of debate.

By Northern blotting, Dhahbi et al. confirmed 5′ *RNY4* fragments in plasma of HD (48), Haderk et al. validated the presence of both *RNY4* full-length and 5′ fragments in CLL exosomes (53), whereas Driedonks et al. showed that EV released from dendritic cells mostly contain full-length *RNY1* and only small amounts of 19–35 nt *RNY1* fragments (58). In this regard, Driedonks and Nolte-'t Hoen suggested that YRNA secondary structures might impede full-length cDNA synthesis, leading to overestimation of fragmented non-coding RNA in sequencing data (59). Indeed, in a RNA-seq study, Godoy et al. detected fragments derived from both the 5′ and 3′ arms (60) whereas other works reported mostly full-length YRNAs (61, 62).

To verify if full-length *RNY4* might be responsible for the differential expression detected by small RNA-seq, not validated by RT-PCR specific from *RNY4*-5′F, we quantified the full-length *RNY4* in 12 ALCL and 12 HD plasmatic exosomes by qRT-PCR

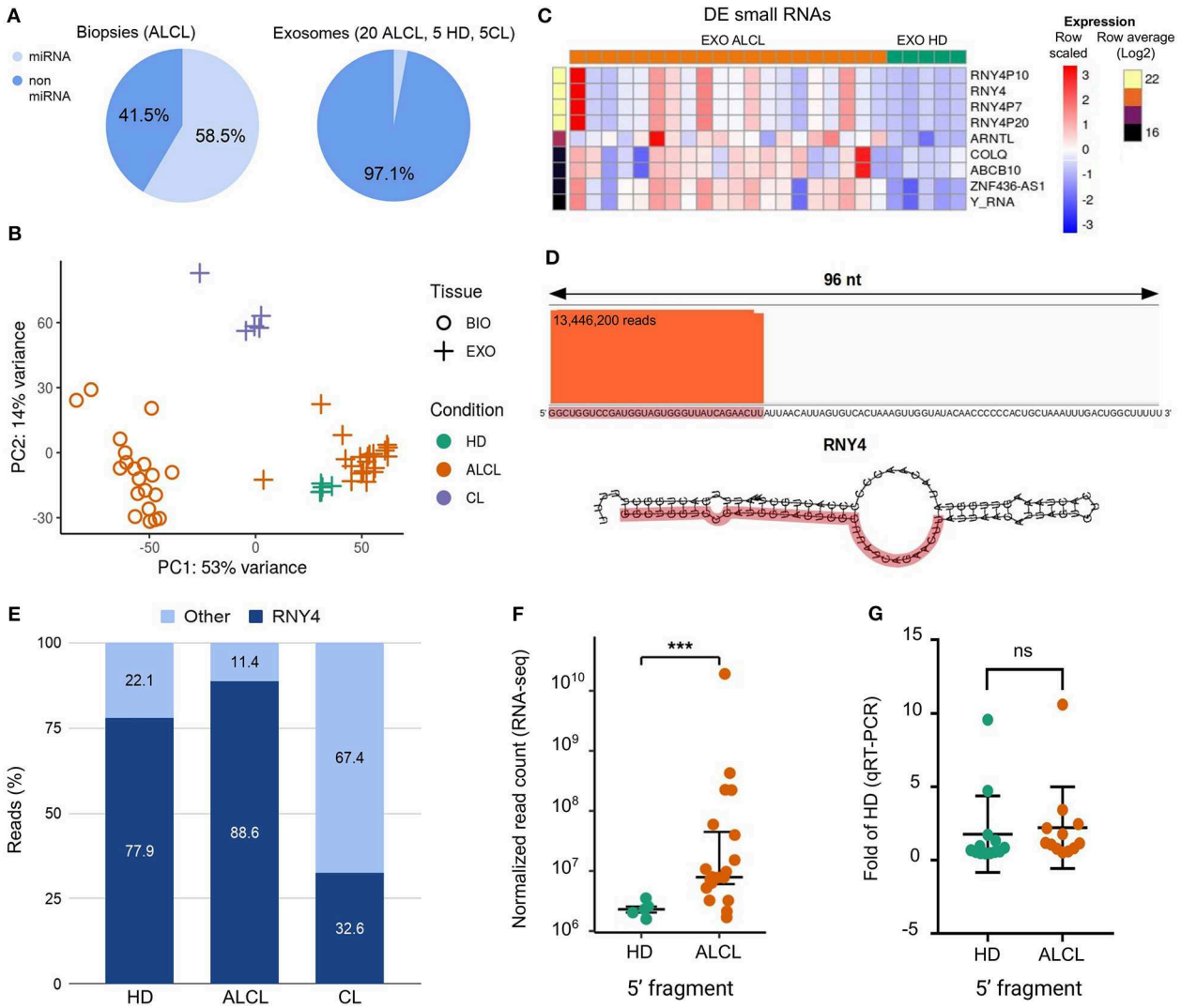

FIGURE 1 | Characterization of exosomal small RNAs in ALCL patients. **(A)** Average proportion of sequence reads aligned to and outside miRNA precursors in anaplastic large cell lymphoma (ALCL) primary tumor biopsies, and in exosomes from ALCL patients, healthy donors (HD) and ALCL cell lines (CL). **(B)** Principal component analysis computed on expression profiles of the 1,007 most abundant (top 5% of expression in at least one sample) non miRNA-derived sRNAs. **(C)** Heatmap of expression profiles in exosome samples of the nine sRNAs most abundant (top 1%) and most varied comparing ALCL and HD (LFC>2). **(D)** Sequence reads alignment to *RNY4* shows reads stacked on the *RNY4* 5′ end; *RNY4* predicted secondary structure is displayed below, highlighting in red the 32 nt fragment identified through RNA-seq. **(E)** Average percentages of non-miRNA RNA-seq reads from exosomes samples mapping to *RNY4* locus and to other putative sRNAs. **(F)** Box-plot of *RNY4*-5′F expression in HD ($n = 5$) and ALCL ($n = 20$) exosomes, according to RNA-seq data. **(G)** Box-plot of *RNY4*-5′F expression measured by qRT-PCR in HD ($n = 13$) and ALCL ($n = 12$) exosomes [***DESeq2 p.adj < 0.001 independent samples from those shown in **(F)**, expression in ALCL relative to average in HD; comparative delta Ct method ($2^{-\Delta\Delta Ct}$); miR-26a-5p used as endogenous control; Mann-Whitney].

using primers from Tolkach et al. (63). The full-length *RNY4* was significantly more abundant in ALCL than in HD (Mann Whitney, $p = 0.017$) (**Figure 2A**).

Further, we considered that full-length YRNAs were mainly reported as contained in exosomes (59), whereas fragments were previously validated by Northern blotting in total plasma and in exosomes (48, 53). We thus investigated the amount of full-length *RNY4* and the *RNY4*-5′F, also distinguishing RNA loaded into exosomes and freely circulating in plasma (RNA of six HD and six ALCL from both exosomes and the column flow-through after the exosome binding step). Of note, most of the full-length *RNY4* was inside exosomes (5.6 times more abundant inside, on average) of both HD (Mann-Whitney $p = 0.009$) and ALCL patient ($p = 0.002$) samples, whereas the fragment was present at a similar level in exosomes and as free circulating RNA (**Figure 2B**). Taken together, these results suggested that *bona fide RNY4* fragments circulate in plasma both enclosed in membranes and as free RNAs, in amounts not discriminating ALCL and HD, whereas full-length *RNY4* is mainly enclosed in exosomes, where it is significantly enriched in ALCL patients.

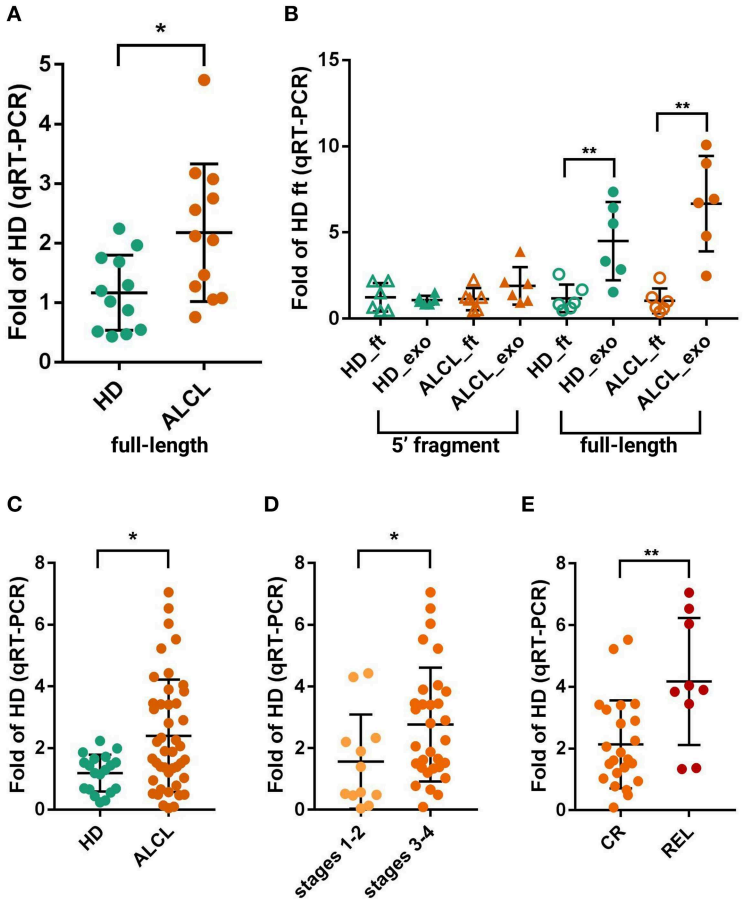

FIGURE 2 | Quantification of *RNY4*-5′F and full-length *RNY4* by qRT-PCR. **(A)** Expression of full-length *RNY4* in 12 HD and 12 ALCL plasmatic exosomes measures by qRT-PCR. **(B)** Expression of *RNY4*-5′F (5′ fragment) and full-length *RNY4* loaded into exosomes (exo) and freely circulating in plasma (flow-through, ft); expression in ALCL relative to average in HD_ft. Full-length *RNY4* was significantly more abundant in both HD and ALCL exosomes than as free circulating RNA (6 HD, 6 ALCL), whereas the *RNY4*-5′F was almost equally distributed inside exosomes and as free circulating sRNA in both HD and ALCL. **(C)** Evaluation of full-length *RNY4* in exosomes from an extended cohort of 44 ALCL and 19 HD confirmed a significantly increased expression of the full-length form in ALCL compared to HD. Full-length *RNY4* was significantly more abundant in patients with stage 3–4 disease compared to those in stages 1–2 **(D)** and also among stage 3–4, in relapsed patients (REL) compared to those in stable complete remission (CR) **(E)**. For panels **(A,C,D,E)**, expression in ALCL has been calculated relative to average in HD; comparative delta Ct method ($2^{-\Delta\Delta Ct}$), miR-26a-5p as endogenous control, Mann-Whitney test for sample comparison were used for all panels. (*$0.01 < p < 0.05$, **$p < 0.01$).

A further examination of an extended cohort of 44 ALCL and 19 HD plasmatic exosomes confirmed the upregulation of the full-length form in ALCL samples (Mann Whitney, $p = 0.017$) (**Figure 2C**).

FULL-LENGTH *RNY4* LOAD IN EXOSOMES OF ALCL PATIENTS CORRELATES WITH DISEASE AGGRESSIVENESS

Next, the extended cohort was further examined considering clinical data. Of importance, *RNY4* abundance correlated with ALCL patients' clinical characteristics. The full-length *RNY4* was more abundant in exosomes of ALCL patients with advanced disease stages (32 ALCL in 3–4 stage vs. 12 ALCL in 1–2 stage; Mann Whitney, $p = 0.049$) (**Figure 2D**). Since most (9/10) of the relapsed patients were diagnosed in stages 3–4 (**Figure 2D**), we analyzed *RNY4* amount at diagnosis in relation to relapse, considering only advanced stages. Compared to cases in stable complete remission ($N = 23$), relapsed patients ($N = 9$) presented at diagnosis with increased levels of exosomal full-length *RNY4* (Mann Whitney, $p = 0.0065$) (**Figure 2E**).

These findings indicate exosomal *RNY4* as a promising biomarker of disease aggressiveness in ALCL, to be quantified with a simple and non-invasive liquid biopsy. Moreover, our data and literature evidence collectively encourage further investigation to ascertain a possible functional role of *RNY4* in ALCL disease aggressiveness, as well as in other lymphoproliferative diseases or different malignancies. Indeed, *RNY4* delivery by CLL exosomes has been recently shown to induce key leukemia-associated phenotypes in monocytes, such as the release of pro-tumorigenic cytokines (CCL2, CCL4, and IL-6) and the expression of the immunosuppressive protein PD-L1, thus generating a tumor-supporting microenvironment

(53). ALCL tumors are characterized by variable histological patterns, mostly depending on tumor cell size and the presence of a large number of reactive histiocytes in the background (64). The biological functions of YRNAs could be multidirectional and we speculate that the association between ALCL and changes in these circulating YRNA reflects some aspects of either the biology of the tumor or the immunosystem reaction of the individual to the tumor. In particular, our results pave the way for investigating the role of *RNY4* as mediator of immunoescape in lymphoma patients. The treatment of monocytes *ex vivo* with tumor exosomes, the uptake as well as exosome-mediated responses by flow cytometry, or cytokine quantification can be used in the next future to elucidate this intriguing aspect.

In conclusion, *RNY4* is a massively loaded molecule in exosomes of ALCL patients, with *RNY4* significantly increased in patients compared to controls. Notably, significantly higher *RNY4* levels were observed in patients diagnosed at advanced stages, and among them, in those that later relapsed. These findings, in the light of available functional data on exosomal *RNY4,* encourage further study of *RNY4* involvement in ALCL tumor microenvironment and disease aggressiveness.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study, these can be found in the NCBI Gene Expression Omnibus (GSE144781).

ETHICS STATEMENT

The study was approved by the ethics committee for clinical experimentation of the Padova hospital CESC (Comitato Etico per la sperimentazione clinica azienda ospedaliera di Padova). Written informed consent was obtained from patients and/or their legal guardians in accordance with the institution's ethical review boards.

AUTHOR CONTRIBUTIONS

FL designed the RNA-seq experiment and the experimental work, analyzed data, and wrote the manuscript. PD performed RNA-seq data analysis and qRT-PCR, and contributed to wrote the manuscript. EG contributed to RNA-seq data analysis and revised the manuscript. PD, FL, and EG prepared figures. CD, AG, and IG processed clinical samples and performed qRT-PCR. EC and MP collected clinical data and commented on manuscript. AB revised the manuscript. SB conceived the study, supervised the bioinformatics work, contributed to experimental results interpretation, and wrote the manuscript. LM conceived the study, supervised the experimental work, and revised the manuscript.

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REVIEW
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The Physiopathology of T- Cell Acute Lymphoblastic Leukemia: Focus on Molecular Aspects

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T-cell acute lymphoblastic leukemia/lymphoma is an aggressive hematological neoplasm whose classification is still based on immunophenotypic findings. Frontline treatment encompass high intensity combination chemotherapy with good overall survival; however, relapsing/refractory patients have very limited options. In the last years, the understanding of molecular physiopathology of this disease, lead to the identification of a subset of patients with peculiar genetic profile, namely "early T-cell precursors" lymphoblastic leukemia, characterized by dismal outcome and indication to frontline allogeneic bone marrow transplant. In general, the most common mutations occur in the NOTCH1/FBXW7 pathway (60% of adult patients), with a positive prognostic impact. Other pathogenic steps encompass transcriptional deregulation of oncogenes/oncosuppressors, cell cycle deregulation, kinase signaling (including IL7R-JAK-STAT pathway, PI3K/AKT/mTOR pathway, RAS/MAPK signaling pathway, ABL1 signaling pathway), epigenetic deregulation, ribosomal dysfunction, and altered expression of oncogenic miRNAs or long non-coding RNA. The insight in the genomic landscape of the disease paves the way to the use of novel targeted drugs that might improve the outcome, particularly in relapse/refractory patients. In this review, we analyse available literature on T-ALL pathogenesis, focusing on molecular aspects of clinical, prognostic, and therapeutic significance.

Keywords: T-cell acute lymphoblastic leukemia, genome, molecular, target therapies, early T cell precursors acute lymphoblastic leukemia

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INTRODUCTION

T-cell acute lymphoblastic leukemia/lymphoma (T-ALL/-LL) is an aggressive hematological tumor, driven by malignant transformation and expansion of T-cell progenitors. T-ALL and T-LL are distinguished by the presence of more or <20% marrow blasts, respectively (1, 2). The *2016 revision* of *WHO classification* added a provisional entity called *Early T-cell precursor* (ETP) *ALL*. This subset is characterized by a unique immunophenotypic (reduced expression of T-cell markers, CD1a, CD8, and CD5) and genetic profile, indicating only limited early T-cell differentiation, with retention of some myeloid and stem cell characteristics (2).

Current treatment of T-ALL consists of high intensity combination chemotherapy, resulting in high overall survival, with the best outcomes observed in pediatric patients (3). Despite the high response rates after first-line therapy, about 20% of pediatric and 40% of adult patients will relapse (4). Differently from B-cell precursors ALL, where highly effective monoclonal antibodies as well

as CD19 targeting chimeric antigen receptor (CAR) T-cells have been developed, in T-ALL only the purine nucleoside analog nelarabine is licensed for relapsed/refractory patients (1, 5). Relapsed/ refractory T-ALL treatment is therefore an unmet need and only new targeted drugs will have the potential to overturn the outcome of these patients.

The purpose of this review is to analyse available data on T-ALL pathogenesis, starting with a brief description of current T-ALL classification and treatment, and then focusing on molecular aspects of clinical, prognostic, and therapeutic significance.

RESULTS

Snapshot on T-ALL Diagnosis, Classification, and Therapy

Diagnosis of T-cell ALL relies on a combination of morphology, immunophenotype, and cytogenetic features, many of which inform prognosis and treatment choices. The morphological distinction between L1 and L2 blasts has now lost clinical relevance since more precise immunophenotypic categories have been set. One of the most widely used is the European Group for the Immunological Characterization of Leukaemias subclassification based on the various stages of T-cell maturation (6). T-lymphoblasts are TdT+ and show positivity for cytoplasmic CD3, the only lineage specific marker. The variable expression of CD1a, CD2, CD4, CD5, CD7, and CD8 distinguishes pro-, pre-, cortical, and mature T-ALL. As regards the relationship between immunophenotype and prognosis, the best outcomes have been observed in the cortical T-cell ALL, while CD1a-negative patients show an increased relapse rate and a lower survival (7, 8). Noteworthy, ETP-ALL is a novel subcategory of T-ALL, characterized by a distinct gene expression profile and immunophenotype. ETP-ALL cells are tipically CD7+ but CD1a– and CD8–, CD5 weak, and express >1 myeloid or stem cell marker (i.e., CD34, CD13, or CD33). These cells originate from a subset of immature thymocytes directly derived from hematopoietic stem cells, thus able to differentiate into both T- and myeloid cells. ETP-ALL accounts for 15% of all T-cell ALL in children and about 35% in adult T-cell disease (9, 10).

As occurs in B-cell ALL, also in T-cell ALL prognosis is influenced by cytogenetics. In a large trial cytogenetic analysis displayed an abnormal karyotype in 72% of patients, with complex karyotypes ($\geq$5 abnormalities) in about 8% of cases, significantly impacting on prognosis (5-year OS 19 vs. 51%, $p = 0.006$) (11). An increasing number of molecular abnormalities have been associated with T-cell ALL and will be discussed in a dedicated paragraph.

First Therapy Line

Regarding therapy, in the first-line setting, the standard of care for fit patients consists of ALL-based pediatric-inspired regimens, incorporating induction (combination of steroids, anthracyclines, and vincristine), consolidation, delayed intensification, and maintenance with central nervous system (CNS) prophylaxis (12, 13). Addition of the enzyme l-asparaginase, and more recently its pegylated *E. coli*-derived form (PEG-ASP), characterized by longer half-life and less anti-drug antibody formation, has been demonstrated to significantly improve response rates and OS both in pediatric (14) and adult patients (15, 16). As occurs in B-cell ALL, indication to allogenic hematopoietic stem cell transplant (alloHSCT) in T-ALL in first remission is based on high risk features at diagnosis and is more and more frequently MRD-driven (17). CNS involvement at diagnosis is more likely in T- than in B-cell ALL (9.6 vs. 4.4%; $p = 0.001$) and has been associated with inferior 5-year OS due to an increased risk of both systemic and CNS relapse (18). The most common prophylaxis employed is the combination of high-dose IV methotrexate and intrathecal chemotherapy (7, 11). A randomized trial stressed the importance of the use of 5 g/sq.m. in T-ALL, higher than those used in B-cell ALL (19). As regards ETP-ALL, a Spanish multicentre study showed the worse prognosis to be ascribed to a lower response to induction therapy than to an increased relapse rate, suggesting that use of different schedules, such as fludarabine, cytarabine, G-CSF, idarubicin (FLAG-IDA), and other more myeloid-oriented chemotherapies, or FLT3-targeted therapies, may play an advantage in this subcategory of patients (20). Current consolidation strategies comprise a delayed intensification including drugs used in induction phase, followed by a 2-year maintenance with 6-mercaptopurine and methotrexate, pulses of vincristine and steroids, and additional IT CNS prophylaxis. Molecular-based and flow cytometry-based techniques allow reliable assessment of minimal residual disease (MRD), whose monitoring at precise timepoints is the standard of care for ALL patients treated with curative intent. The molecular method consists of identifying clone-specific rearrangement with Sanger on next-generation sequencing into the immunoglobulin heavy chain gene or T-cell receptor genes by using a large panel of consensus primers, generating patient-specific real-time quantitative polymerase chain reaction assays for quantification in about 90% of cases, with a quantitative range of 10^{-4}. Despite variable definitions of "early" assessment of MRD (from 6 to 10–16 weeks from the start of therapy), plenty of studies in ALL have confirmed that early MRD response is the most powerful predictor of long-term survival in adult patients with ALL (21–23). Finally, myeloablative alloHSCT should be considered for high-risk T-cell disease. Allocation to alloHSCT may vary among study groups, but generally speaking, failure to achieve CR after induction therapy, high white cell count at presentation, high risk cytogenetics/immunophenotype, and MRD persistence at defined timepoints can all be used to allocate to transplant (11, 24, 25). As regards the subcategory of ETP-ALL, two trials demonstrated improvement in survival in ETP-ALL patients transplanted early in case of treatment resistance (20). Considered its better prognosis, consolidation with alloHSCT is not considered necessary in T-LBL, unless suggested by an adverse course of the disease (26).

Relapsed Disease

About 80% of relapses occur within 2 years of diagnosis. With <7% of survival rate at 5 years (27), relapsed T-ALL has dismal outcome, and no standard strategies are available so far. Response rates using standard chemotherapy regimens such as FLAG-IDA are around 30–40%, with a median OS

of 6 months in responders (28). Nelarabine is the only new agent specifically licensed for relapsed/refractory T-cell ALL/LBL. Used as single agent, this drug induced ORR of 14–55% in pediatric patients (29) and 41–46% in adults, with 1-year OS of 28% (30). Neurotoxicity is the major toxicity, affecting around 15% of patients, with more severe and irreversible cases in a minority of patients (31). Importantly, most of the patients obtaining a CR with nelarabine were able to proceed to alloHSCT.

Focus on the Molecular Pathways Involved in T-ALL Pathophysiology

T-ALL results from a multistep transformation process in which the accumulation of genetic alterations affects key oncogenic/tumor suppressors pathways, that are responsible for proliferation, survival and differentiation of T-cells (32, 33). The molecular steps involved in T-ALL pathogenesis encompass: transcriptional deregulation of oncogenes/oncosuppressors, NOTCH1 signaling, cell cycle deregulation, kinase signaling (including IL7R-JAK-STAT pathway, PI3K/AKT/mTOR pathway, RAS/MAPK signaling pathway, ABL1 signaling pathway), epigenetic deregulation, ribosomal dysfunction, and altered expression of oncogenic miRNAs or long non coding RNA (34) (**Figure 1**).

Transcriptional Deregulation of Oncogenes and Oncosoppressors

Among the genetic abnormalities, chromosomal translocations of transcription factor oncogenes to regulatory regions of T-cell receptor (TCR) genes are characteristic of T-ALL (34). Approximately 50% of patients harbor chromosomal translocations involving 14q11 (TCR alfa and TCR delta) and 7q34 (TCR beta) (35). Other mechanisms involved are chromosomal rearrangements with other regulatory sequences, duplication/amplification, and mutations or small insertions generating novel regulatory sequences acting as enhancers (36).

Transcriptional factors belonging to bHLH, LMO, and HOX families are also implicated (**Table 1**). The largest subgroup, representing about 30–35% of T-ALL, is characterized by the abnormal expression of TAL1 (1p32), a bHLH member, which results from either $t_{(1;14)}$ (p32;q11), and $t_{(1;7)}$ (p32;q35) translocations, small insertions, mutations or 1p32 deletion (36, 37). TAL1 expression is associated with a late cortical thymocyte immunophenotype (CD1a-) (38), and correlates with favorable outcomes (35, 39).

LMO1 (11p15) and LMO2 (11p13) are part of a transcriptional complex, and are aberrantly expressed at high levels in ∼15% of T-ALL, due to both translocations to TCR loci and small chromosomal deletions (32, 45, 48, 49). Also these cases carry a favorable prognosis (35).

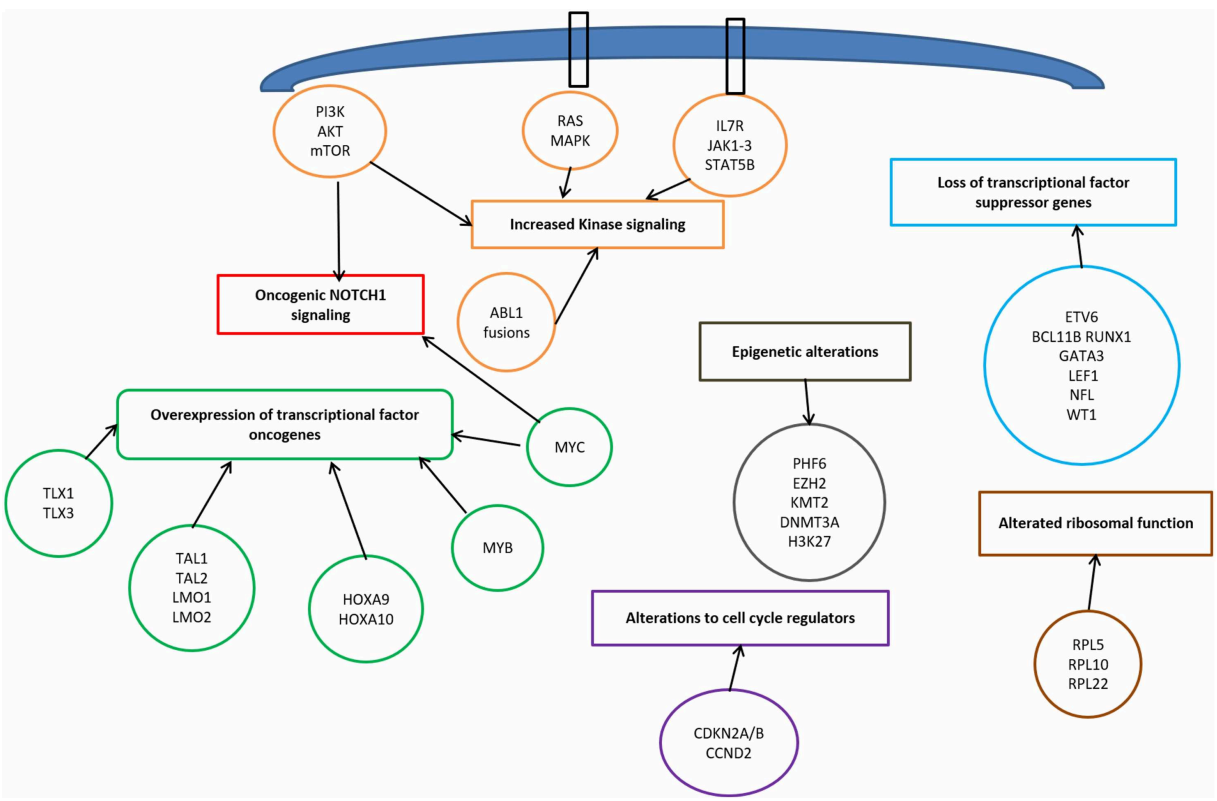

FIGURE 1 | Signaling pathways involved in T-cell acute lymphoblastic leukemia pathophysiology.

TABLE 1 | Molecular pathways involved in T-ALL pathogenesis.

Gene	Locus	Type of mutation	Frequency	Relevance	References
TRANSCRIPTION REGULATOR (ONCOGENE)					
TAL1	1p32	Aberrant expression due to translocations involving one of the TCR gene [TCRalfa (14q11) or TCRbeta (7q34)]; duplications or amplifications; mutations or insertions;	30–35%	Favorable outcome	(35–39) (40, 41)
TAL2	10q24		Rare	–	
TLX1/HOX11	10q24		5–10% (children), 30% (adults)	Favorable outcome	(32, 35, 38, 39, 42–47)
TLX3/HOX11L2	5q35		20–25% (children), 5% (adults)	Poor outcome	
LMO1	11p15	Aberrant expression due to t (11,14) or small deletion	15%	Favorable outcome	(32, 35, 45, 48, 49)
LMO2	11p13				
HOXA9;HOXA10	7p15	Chromosomal translocations and inversions involving TCRs loci	3%	–	(32)
NKX2-1;NKX2-2	14q13; 20p11		5% (children)	–	
MLL	11q23	Rearrangements with various partners	5% (children)	Poor outcome	(33)
MYC	8q24	Mutations or rearrangements or amplifications, rarely t (8,14)(q24;q11)/MYC-TCRalfa	6%	Subclonal; poor outcome; more common in T-LL	(32, 50–54)
MYB	6q23		10%	–	(45, 55)
TRANSCRIPTION REGULATOR (ONCOSOPPRESSOR)					
BCL11B	14q3	Deletions or inactivating mutations	10%	–	(32, 56–60)
ETV6*	12p13		13% (25% of ETP)	In etp, poor outcome	
RUNX1*	21q22		10–20% (most in ETP)	In ETP, poor outcome	
GATA3*	10p14		5% (most in ETP)	In ETP, poor outcome	
LEF1	4q24		10–15%	IF early T cortical	
WT1	11p13		10%	–	
NF1*	17q11		More common in children	–	
NOTCH1 SIGNALING					
NOTCH1	9q34.3	activating mutations most of all; t (7,9)(q34;q34)/TCRbeta-NOTCH1 in < 1% of cases	60–70%	Favorable outcome; NOTCH inhitors	(33, 51, 61–69)
FBXW7	4q31.3	Loss of function mutations	15%	Prognostic if evaluated in combination with NOTCH1	
CELL CYCLE REGULATION					
CDKN2A (p16INK4A; p14ARF); CDKN2B (p15)	9p21	Deletions	70%	–	(32, 70, 71)
CDKN1B (p27KIP1)	12p13	Deletions	12%	–	
CCND2(cyclin D2)	12p13	Chromosomal translocations involving TCRs loci	3%	–	
RB1	13q14	Deletions	15%	–	
IL7-JAK-STAT PATHWAY					
IL7R*	5p13	Activating mutation	20–30% (most in ETP): JAK3 16%; JAK1 10%; IL7R 10%; STAT5B 5–10%	–	(33, 34, 36, 72–75)
JAK1*	1p32.3-p31.3	Gain of function mutations		poor outcome; JAK inhibitors	
JAK2	12p13	Translocation t (9,12)(p24;p13) involving ETV6-JAK2		–	

(Continued)

TABLE 1 | Continued

Gene	Locus	Type of mutation	Frequency	Relevance	References
JAK3*	19p13-p12	Gain of function mutations		in ETP, poor outcome	
STAT5B	17q21.2	Gain of function mutations		–	
DNM2	19p13.2	Loss-of-function		–	(76)
PTPN2	18p11.3-p11.2	Inactivating mutations	6%	–	
PTPRC (CD45)	1q31.3-q32.1	Inactivating mutations		–	
PIM1	6p21	t (6, 7)(p21; q34)/PIM1-TCRbeta	5%	–	
PI3K-AKT-mTOR PATHWAY					
PI3K	3q26	Gain of function mutations	5%	PI3K inhibitors	(77–80)
AKT	14q32	Gain of function mutations	2%	–	
PTEN	10q23	Loss of function mutations, deletions	10-15%	–	
mTOR	1p36.22	Gain of function mutations	<1%	mTOR inhibitors	
RAS PATHWAY					
RAS (N-RAS, K-RAS, H-RAS)*	1p13; 12p12; 11p15	Activating mutations	Most in ETP	Poor outcome	(34, 35, 66, 74, 81)
NF1*, PTPN11	17q11; 12q22	Loss of function mutations	Most in ETP	in ETP, poor outcome	(82, 83)
ABL KINASE SIGNALING					
ABL1	9q34	Rearrangements, episomal amplifications (NUP214-ABL1; EML1-ABL; ETV6-ABL)	8%	TK inhibitors	(35, 84–86)
EPIGENETIC REGULATION					
PHF6	Xq26	Inactivating mutations or deletions	16% (children), 38% (adults), M>>>F	–	(36, 87)
KDM6A	Xp11		6–7%	–	
EZH2* (and others of PCR2 complex)	7q36		25%	in ETP, poor outcome	
DNMT3A*	2p23		15% (adults), most in ETP	in ETP, poor outcome	
H3K27	1q42			–	
RIBOSOMAL FUNCTION					
RPL5	1p22	Inactivating mutations	2%	–	(32, 88)
RPL10	Xq28	Missense mutations at residue R98	6–8% (children)	Hypoproliferative phenotype	
RPL11	1p36	Inactivating mutations	1%	-	

Genes more commonly involved in ETP-ALL. TK, tyrosine kinase; ETP, early T-cell precursor.

Among HOX genes family, TLX1 (10q24, formerly HOX11), and TLX3 (5q35) are over-expressed in T-ALL. TLX1+T-ALLs represent 30% of adult T-ALLs and result from the translocation $t_{(10;14)}$ (q24,q11); the latter involves the TCR locus (42, 43) and contributes to thymocyte arrest at the early cortical stage (CD1a+), conferring favorable outcome (32, 44, 45). On the contrary, TLX3 overexpression (20–25% of pediatric T-ALL) correlates with a poor outcome; it results from $t_{(5;14)}$ which places this oncogene under the control of T-cell regulatory sequences in the BCL11B locus (32, 35, 39, 46, 47).

Other Protoncogenes Involved: MLL, MYC, and MYB

MLL (11q23), originally described in pediatric acute myeloid leukemia, is also involved in T-ALL pathogenesis. The outcome of MLL-rearranged leukemias is generally unfavorable, however this relationship is less clear in T-ALL. MLL-MLLT1 rearrangement, present in 2–3% of T-ALL, has a better outcome, whereas PICALM-MLLT10 rearrangement (about 6–7% of cases) is linked to worse prognosis (39, 89, 90).

MYC (8q24) and MYB (6q23) are proto-oncogenes involved in the transcriptional deregulation observed in T-ALL. In early T-cell development, MYC plays an important role in the control of cell growth downstream NOTCH1 and TCR signaling (50). Moreover, rearrangements involving PI3K/AKT pathway often result in MYC overexpression (52). The translocation $t_{(8;14)}$, involving the TCR, is present in only 1% of MYC+ T-ALL (53), and other mechanisms occur: translocations involving others partners, duplications, amplifications, and reduced degradation (32). In a subgroup of about 6% of T-ALL,

MYC translocations are secondary abnormalities, present in subclones, and are associated with induction failure, high rate of relapse, and with an aggressive clinical course (52). The genetic profile of these MYC- translocated T-ALL is characterized by concomitant abnormalities, including CDKN2A/B deletions, PTEN inactivation, and mutations typical of myeloid neoplasms, such as DNMT3A (54). Regarding MYB, it is activated in T-ALL harboring the $t_{(6;7)}$ translocation, which is common among children younger than 2 years of age, or as a result of duplications or amplification of 6q23 (45, 55).

In addition to oncogenes, tumor suppressors contribute to transcriptional deregulation in T-ALL, usually due to deletions or inactivating mutations. BCL11B (14q32), ETV6 (12p13), RUNX1 (21q22), GATA3 (10p14), LEF1 (4q24), WT1 (11p13), and NF1 (17q11) are the main oncosoppressors involved (32).

ETV6, RUNX1, and GATA3, described also in acute myeloid leukemia, are deleted or inactivated in ETP-ALL, and correlate with poor outcome: ETV6 (12p13) mutations account for ∼25% of ETP-ALL (56), whilst RUNX1 (21q22), and GATA3 (10p14) mutations are less common. BCL11B (14q32) is mutated in 10% of T-ALL (57); LEF1 (4q24) in 10–15% and is associated with an early cortical thymocyte immunophenotype (58), and WT1 (11p13) in about 10% of cases (59). Monoallelic deletion of 17q12, involving the tumor suppressor NF1, is common in children, but it has been described also in adults (60).

NOTCH1 Pathway

NOTCH1 pathway is essential for T-cell lineage commitment and maturation of hematopoietic progenitors (61). Rarely, the $t_{(7;9)}$ (q34;q34.3) translocation leads to the expression of a constitutively active form of NOTCH1 (9q34.3) (62). However, in over 60% of T-ALLs, NOTCH1 aberrant expression results from activating mutations (63). These mutations lead to ligand-independent cleavage and activation of the intracellular NOTCH1 domain and to the stabilization of the active protein (33). Loss of function of negative regulators of NOTCH1 is an alternative mechanism. As a matter of fact, 10–15% of T-ALL, harbor mutations in FBXW7 (4q31.3), a protein that promotes NOTCH1 proteasomal degradation, and lead to increased NOTCH1 protein stability (64). In prognostic models, patients with NOTCH1 and FBXW7 mutations are defined as low risk cases (65, 66).

NOTCH1 pathway is also a central driver of T-cell metabolism and promotes leukemia cell growth via direct upregulation of anabolic pathways, including ribosome biosynthesis, protein translation and nucleotide and aminoacid metabolism. The effect on cell growth is enhanced by the upregulation of MYC (51, 67, 68). Furthermore, NOTCH1 activates mTOR/Akt pathway and increases the glucose uptake in maturating thymocytes. In summary, oncogenic Notch1 pathway is responsible for enhanced aerobic glycolysis and upregulation of anabolic pathway leading to increased proliferation (69).

Cell Cycle Deregulation

The loss of cell cycle control has a prominent role in the pathogenesis of T-ALL. Deletions of the cyclin-dependent kinase inhibitor 2A (CDKN2A encoding tumor suppressors $p16^{INK4A}$ and $p14^{ARF}$) and 2B (CDKN2B encoding the tumor suppressor $p15^{INK4B}$) loci on 9p21 are present in up to 70% of T-ALL, leading to abnormal proliferation control (70). Moreover, deletions in retinoblastoma 1 (RB1, locus on 13q14), a regulator of cell cycle progression, are found in 15% of T-ALL, and deletions involving the CDKN1B locus (12p13, encoding $p27^{KIP1}$) are present in about 12% loci (32). Finally, high levels of cyclin D (CCND2) are present in 3% of T-ALLs, as a result of translocations with TCR loci (71).

Kinase Signaling Pathways

Kinase signaling pathways aberrantly activated in T-ALL include IL7R/JAK/STAT, PI3K/AKT/mTOR, RAS/MAPK, and ABL kinase signaling (34, 36).

IL7R/JAK/STAT pathway is essential for normal T-cell development and is triggered by the interaction between IL7 and its heterodimeric receptor. Upon ligand-binding, IL7R dimerizes and induces JAK1 and JAK3 phosphorylation, with consequent STAT5 activation. STAT5 dimerizes and translocates to the nucleus, where regulates many target genes, including BCL2 family members (72, 73). Activating mutations of IL7R (5p13), JAK1 (1p32), JAK3 (19p13), and/or STAT5B (17q21) are present in 20–30% of T-ALL cases, with a higher frequency in ETP-ALL patients (33, 74). JAK3 mutations are present in about 16% of T-ALL cases, and a strong association between JAK3 mutations and HOXA9 expression has been demonstrated (75). Furthermore, 6% of T-ALLs are characterized by haplo-insufficiency of negative regulators of this pathway, such as DNM2 (19p13), PTPN2 (18p11), and PTPRC (1q31) (76). The rare $t_{(9,12)}$ (p24;p13) translocation encodes a constitutively active kinase protein, ETV-JAK2, leading to aberrant JAK signaling (91). PIM1 is the ultimate target of the JAK/STAT downstream, and high PIM1 expression is a biomarker of activation of this pathway; PIM1 can be overexpressed also as a result of translocation $t_{(6,7)}$ (p21;q34), involving TCR beta (76).

PI3K/AKT/mTOR pathway is aberrantly activated in T-ALL, resulting in enhanced cell metabolism, proliferation, survival, differentiation, and impaired apoptosis (77). Hyperactivation of this oncogenic pathway is mainly caused by loss-of-function mutations/deletions of PTEN (10q23), occurring in about 10–15% of T-ALLs (78, 79). Additional mutations include gain-of-function mutations in regulatory and catalytic subunits of PI3K (3q26) (4,5% of cases), or in AKT (14q32) or mTOR (1p36) (2 and <1% of cases, respectively) (80).

RAS proteins, including H-RAS (11p15), N-RAS (1p13), and K-RAS (12p12), are fundamental signal transductors from cell surface to downstream effectors (34). RAS-MAPK signaling pathway is frequently hyperactivated in T-ALL, and RAS mutations are present in about 5–10% of cases, particularly in high risk ETP-ALL and in relapsing patients (35, 66, 74, 81). RAS pathway regulators may also be mutated: loss-of-function of NF1 (17q11) and PTPN11 (12q22) have been described in 3% of cases (82, 83).

Finally, ABL1 gene (9q34) is rearranged in 8% of cases, leading to constitutive kinase activity (84). The most frequent rearrangement is NUP214-ABL1 amplification (9q34 amplification), observed in 6% of patients (85), whilst EML1-ABL and ETV6-ABL1 are less common (35). NUP214-ABL is

a secondary, subclonal alteration and has not been linked with poor prognosis (86).

Epigenetic Deregulation

Mutations in epigenetic factors are frequent in T-ALL: PHF6 (Xq26), SUZ12 (17q11), EZH2 (7q36), TET2 (4q24), H3F3A (1q42), KDM6A (Xp11), EED (11q14), SETD2 (3p21), and DNMT3A (2p23) mutations are the most common (32, 35). Considering the most frequent, PHF6 is a histone modifier, involved in transcriptional regulation, DNA damage response and cell cycle control. Loss-of-function mutations or deletions of this gene, exclusively found in male patients, are present in 16% of pediatrics and 38% of adults, and result in G2/M cell cycle arrest. Mutational loss of PHF6 is associated with the aberrant expression of the transcription factor oncogenes TLX1 and TLX3 (87). H3K27 regulates methylation, and together with the PRC2 complex (polycomb repressive complex 2, that includes EZH2, SUZ12, and EED) is mutated in up to 25% of T-ALLs (36).

Ribosomal Function

Ribosomes are cellular components required for protein synthesis, a crucial step in rapidly dividing leukemic cells. Ribosomal genes RPL5 (1p22), RPL10 (Xq28), and RPL11 (1p36) have been described to be mutated in T-ALL (32). RPL10 mutations are found in 6–8% of pediatrics, with the recurrent $RPL10^{R98S}$ mutant allele in most cases (32, 88). $RPL10^{R98S}$ mutant leukemia cells may increase the expression of anti-apoptotic protein BCL2. RPL10 R98S mutations are mutually exclusive with JAK/STAT mutations and are associated with a hypoproliferative phenotype (88).

Novel Therapeutic Strategies

Regarding therapy, T-ALL is an aggressive leukemia with limited options, particularly in the relapsed/refractory setting. A better understanding of T-ALL pathogenesis may allow the development of molecular targeted therapies (**Table 2**) (49). For instance, the high prevalence and prominent role of NOTCH1 mutations make it a promising therapeutic target. Clinical trials have explored the use of γ-secretase inhibitors (86), with limited efficacy and gastrointestinal toxicity (92) that can be reduced by the addition of steroids (93, 104). An example is PF-03084014 that has been tested in a clinical study of relapsed/refractory T-ALL/T-LL (A8641014), with one out of 8 patients experiencing complete response lasting about 3 months (94). Other options are NOTCH transcriptional complex inhibitors or antibodies against NOCTH1 (105). Cell cycle dysregulation by CDK4/CDK6 altered pathway is another potential target, and CDK4/CDK6 inhibitors (86) such as palbociclib recently entered clinical trials. The constitutive activation of PI3K/AKT/mTOR signaling pathway may also be targeted: several PI3K inhibitors showed anti-leukemic effects in T-ALL cell lines, whereas mTOR inhibitors seem to prolong survival in T-ALL cells (34). The most studied molecules were everolimus and temsirolimus (106), that induced variable responses (0–50%) in association to chemotherapy and in a small number of cases (94–96). The limited efficacy of mTOR inhibitors seems to be linked to the activation of compensatory signaling pathways (106). Furthermore, dual PI3K/mTOR inhibitors, NVP-BEZ235 and NVP-BKM120, have been studied. BEZ235 had antiproliferative and proapoptotic effect in T-ALL cell lines (97), and a clinical trial has been started (NCT01756118). BKM120/Buparlisib showed modest efficacy and was tolerable in advanced acute leukemia (only 1 patient with T-ALL) in a recent clinical trial (98). As regards cytokine signaling, JAK-STAT pathway is activated in T-ALL

TABLE 2 | Clinical and preclinical trials with target therapies in T-cell acute lymphoblastic leukemia.

Type of study	Molecule	Reference
NOTCH1 INHIBITORS		
Clinical, phase 1	MK-0752	(92)
Preclinical	PF-03084014 + DEX	(93)
Clinical	PF-03084014	(94)
Clinical, phase 1b/2	Crenigacestat (LY3039478) + Dex	NCT02518113
Clinical, phase 1	BMS-906024 alone or + DEX	NCT01363817
Clinical, phase 1	BMS-906024	(95)
Clinical, phase 1	MK0752-013	NCT00100152
Clinical, phase 1/2	RO4929097	NCT01088763
CDK4/6 INHIBITORS		
Preclinical	LEE011 + a panel of drugs	(96)
Clinical, phase 1	Palbociclib + CT	NCT03792256/ AINV18P1
PI3K/mTOR DUAL INHIBITORS		
Preclinical	NVP-BEZ325/ Dactolisib	(97)
Clinical, phase 1	NVP-BEZ325/ Dactolisib	NCT01756118
Clinical, phase 1	NVP-BKM120	(98)
mTOR INHIBITORS		
Clinical, phase 1	Everolimus (RAD001) + CT	NCT01523977
Clinical, phase I/II	Everolimus + HyperCVAD	(99)
Clinical, phase I	Temsirolimus (CCI799) + UK ALL R3 (Dex+Mitox+VCR+pegAsp)	(100)
Clinical, phase I	Everolimus + CT (VCR, PDN, peg Asp, Doxo)	(101)
Clinical, phase II	Sapanisertib	NCT02484430
Clinical, phase 1	Sirolimus + HyperCVAD	NCT01184885
Clinical, phase 1	Temsirolimus + VP16 + CTX+ DEX	NCT01614197
Clinical, phase 1	Everolimus + Nelarabina+ CTX+ VP16	NCT03328104
TK INHIBITORS		
Clinical, phase 1/2	Ruxolitinib (doses ranging from 10–80 mg) + L-ASP, VCR, and PDN	NCT03613428
Preclinical	Imatinib or Dasatinib or Nilotinib	(102)
Clinical	Imatinib + CT	NCT00049569
HDAC INHIBITORS (EPIGENETIC REGULATORS)		
Clinical	Chidamide + CT	NCT03564704
	BCL2 inhibitors	
Clinical	Venetoclax + CT	(103)
Clinical, phase 1/2	Venetoclax + low intensity CT	NCT03808610
Clinical, phase 1b/2	Venetoclax + Vincristine	NCT03504644

CT, chemotherapy; Dex, dexamethasone; VCR, vincristine, Mitox, mitoxantrone; Asp, asparaginase; Doxo, doxorubicine; VP16, etoposide; CTX, cyclophosphamide; PDN, prednisone; TK, tyrosine kinase; HDAC, histone deacetylase.

and about 5% of cases are driven by tyrosine kinase oncogene fusions, particularly the NUP214-ABL1 rearrangement (86). JAK inhibitors, such as Ruxolitinib and Tofacitinib, have been studied in preclinical models with activation of IL7R/JAK/STAT pathway (34, 86). In addition, imatinib, dasatinib, and nilotinib are all active against *NUP214-ABL1*-positive T-cells, with different ability to inhibit this kinase and induce apoptosis in preclinical studies (102). Finally, $RPL10^{R98S}$ mutant leukemia cells are potentially sensitive to Bcl2 inhibitor venetoclax (88). Venetoclax combined to chemotherapy induced a morphological remission in 60% of patients (including ETP-ALL) in a recent retrospective study (103).

DISCUSSION AND FUTURE PERSPECTIVES

T-ALL is a genetically heterogeneous disease caused by a multistep process, involving cell growth, proliferation and differentiation of T-cells (36, 66). A better understanding of the molecular physiopathology may refine classification and prognostication. Regarding the former, molecular findings allowed the definition of the ETP-ALL subgroup, characterized by a distinct gene expression profile and immunophenotype (9). Moreover, high frequencies of FLT3, NRAS/KRAS, DNMT3A, IDH1, and IDH2 mutations have been found in ETP-ALL (107), similarly to what observed in myeloid leukemic stem cells. This new entity is associated with high levels of minimal residual disease after induction chemotherapy (10) and inferior long-term outcomes (25, 108). Beyond ETP-ALL, other recurrent mutations carry prognostic significance. Among them, the most common occur in the NOTCH1/FBXW7 pathway (60% of adult patients) (63), and confer a positive prognosis in most studies (65, 109, 110). A risk classification based on the presence or absence of NOTCH1/FBXW7, PTEN, or N/K-RAS mutations has been proposed (111). The good-risk group (significantly superior OS and inferior cumulative incidence of relapse) harbored mutations in the NOTCH1/FBXW7 pathway with no associated mutations in PTEN or N/K-RAS; mutated NOTCH1/FBXW7 genes plus mutations in PTEN or N/K-RAS were classified as poor risk with OS 44% and cumulative incidence of relapse 54%.

The study of genetic lesions involved in T-ALL pathogenesis may lead to the development of new targeting drugs. In particular, different inhibitors of NOTCH1 pathway are under active study, including γ-secretase inhibitors, blocking of NOTCH transcriptional complex, and antibodies against NOCTH1. Cell cycle blockers like palbociclib and PI3K-, mTOR- and dual inhibitors (everolimus and temsirolimus, NVP-BEZ235 and NVP-BKM120), showed promising anti-leukemic effect both *in vitro* and *in vivo*. Tyrosine kinase inhibitors targeting IL7R/JAK/STAT pathway (ruxolitinib and tofacitinib) and NUP214-ABL1-mutated ALL (imatinib, dasatinib and nilotinib) are all active against T-cell blasts. Finally, Bcl2 inhibitor venetoclax may have a role in $RPL10^{R98S}$ mutant ALL.

In conclusion, in the last years the better understanding of genetic lesions in T-ALL paved the way to novel target therapies, and many preclinical and clinical trials are ongoing. However, the rarity of the disease makes it hard to design specific trials, and the complexity of the molecular landscape may account for the limited efficacy of selective inhibitors in clinical studies. In this setting, differently from other leukemic contexts where chemo-free regimens are emerging (as observed for Ph+ B-ALL targeted with TK-inhibitors and bispecific antibodies), combination chemotherapy is still needed to establish a response. Nevertheless, the inhibition of more ancillary targets like Bcl2 seems to evoke better anti-leukemic effect and may lead the way for future studies and combinations.

AUTHOR CONTRIBUTIONS

BF, JR, and JG wrote the paper and revised it for intellectual content. NF designed the study and revised the paper for intellectual content. LB revised the manuscript for intellectual content. All authors made substantial contributions to the conception or design of the work, revised it critically for important intellectual content provided approval for publication of the content, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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The Tumor Microenvironment of DLBCL in the Computational Era

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Among classical exemplifications of tumor microenvironment (TME) in lymphoma pathogenesis, the "effacement model" resembled by diffuse large B cell lymphoma (DLBCL) implies strong cell autonomous survival and paucity of non-malignant elements. Nonetheless, the magnitude of TME exploration is increasing as novel technologies allow the high-resolution discrimination of cellular and extra-cellular determinants at the functional, more than morphological, level. Results from genomic-scale studies and recent clinical trials revitalized the interest in this field, prompting the use of new tools to dissect DLBCL composition and reveal novel prognostic association. Here we revisited major controversies related to TME in DLBCL, focusing on the use of bioinformatics to mine transcriptomic data and provide new insights to be translated into the clinical setting.

Keywords: tumor microenvironment, transcriptomics, deconvolution, prognostication, DLBCL

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INTRODUCTION

Diffuse large B cell lymphoma (DLBCL) has long been regarded as a paradigm of aggressive diseases composed of malignant B cells dividing rapidly and independently of stimuli from the surrounding tumor microenvironment (TME) (1). Over the last few years, evolving technologies enabling deeper genomic and transcriptomic profiling revealed an underestimated complexity of DLBCL biology, involving both the malignant and non-malignant compartments of the disease.

Seminal gene expression profiling (GEP) studies showed striking associations between expression of genes reflecting tumor cell-of-origin (COO) and outcomes to standard immuno-chemotherapy (2). Two distinct molecular categories, germinal center B cell and activated B cell (ABC), were incorporated in the revised WHO classification of DLBCL (2). The development of immunohistochemistry (IHC) algorithms to surrogate GEP was promptly followed by the commercialization of a gene panel for proper COO determination (3). The assessment of the transcriptional, rather than phenotypical, features of DLBCL also resulted in a remarkable improvement of survival prediction. With the advent of new technologies, such as NanoString, the digital gene expression measurement on formalin-fixed paraffin-embedded (FFPE) biopsies facilitated the inclusion of COO categorization in daily clinical practice (3). However, very recent integrative analyses by whole-exome and transcriptome sequencing brought DLBCL genetics to a new level (4–6), identifying molecular categories within COO classification characterized by distinct drivers with novel prognostic and therapeutic implications.

While a substantial amount of information from these studies is being translated into the clinic, results capturing the molecular aspects of TME are still under debate. Historical GEP

analyses provided alternative categorization of DLBCL based on the differential expression of genes reflecting inflammatory host response and oxidative metabolism (7) or enrichment in peculiar immune and extra-cellular determinants (8). Such observation remained poorly applied on clinical ground owing to an incomplete comprehension of the specific cellular/molecular TME determinants and the precise mechanisms of their prognostic impact. In a recent translational effort, our group exploited a computational approach to reinterpret large transcriptional data and provide a pure TME-based prognosticator that improves the COO risk stratification (9). Latest results from sequencing studies and clinical trials on new drugs (i.e., lenalidomide and ibrutinib) underscored the relevance of studying DLBCL heterogeneity, taking into proper account the impact of TME in diagnostics, prognostics, and therapeutic prediction.

We reviewed current controversies related to TME in DLBCL, with particular emphasis on recent computational strategies capturing new microenvironmental features, at both the cellular and the molecular levels.

EVOLVING TECHNOLOGIES TO FACE TME-RELATED CONTROVERSIES

About 20 years ago, the first GEP-scale analysis of *de novo* nodal DLBCL not otherwise specified demonstrated that morphological approaches, even supplemented by IHC, were incapable of capturing divergent molecular modules between tumors, and identified two main subgroups resembling the diverse stage of B cell differentiation with a different prognosis (10). Each subgroup also showed consistent transcriptomic heterogeneity of non-malignant compartment. The expression level of many genes reflected a variable extent of T cell (*TCR-beta, CD3e, Fyn, LAT, PKC-u*), monocyte/macrophages (*CD14, CD105, CSF-1R, FcR-gamma*), and natural killer (*NK4*) infiltration as well as extracellular matrix (ECM) remodeling by metalloproteinases (i.e., *MMP9* and *TIMP*), integrins, chemokines, and other stromal axes (i.e., CXCR4/SDF-1). Assembled in the so-called lymph node (LN) signature, these genes were shared by samples of normal lymph nodes and tonsil (10), remarking their structural and immune function within secondary lymphoid organs (SLO) (11). A second large genome-scale study highlighted a direct correlation between the expression of the LN signature and a better outcome after CHOP chemotherapy, emphasizing that the ABC subgroup had the lowest enrichment of genes in the signature (12). Their expression was also inversely related to a "proliferation" signature including genes regulating malignant growth processes and *BMP-6*, a single TGF-related mesenchymal gene associated with poor outcome. Once again, stromal factors implicated in ECM organization and shared by elements of innate immunity, especially macrophages (Mo), dendritic cells (DC), and NK, were involved in the physiopathology and drug response of DLBCL. This observation was partially confirmed by Monti et al. (7) who identified a "host response" gene set in DLBCL, showing a coordinated activation of inflammatory response driven by $CD3^+$ T cells, DC, Mo, and NK, adhesion axes (LFA-1, PECAM-1, and SDF-1), cytokine/chemokine stimuli (especially IFN and TNFα), and ECM components (i.e., collagens). However, the patients in this cluster did not show any therapeutic advantage following CHOP chemotherapy. A subsequent work by Lenz et al. (8) definitely recognized a "stromal" signature related to the sorted CD19-negative non-malignant component, reflecting high deposition of ECM proteins, as fibronectin (FN), secrete protein acid rich in cysteine (SPARC), and various collagen isoforms and prevalent infiltration of cells of myelo-monocytic lineages. DLBCL expressing this signature showed longer survival after R-CHOP independently of COO, suggesting an intriguing stromal protection and raising the question on whether the abundance of histiocytes prompts the tumor cell killing by rituximab.

Beyond their relevance in characterizing the tumor cell fraction, GEP results strengthened the idea that finely regulated interconnections between mesenchymal (stromal) and hematopoietic (immune) counterparts in SLO govern the extent of inflammatory reactions as the tumor evolves. Such underestimated mechanisms were likely independent of COO and seemed to underlie the inter-patient diversity in drug responsiveness. Measuring selected TME genes by RNA microarrays, however, remained mechanistically uninformative and, although of certain prognostic utility, was hampered by cost, standardization issues, and scarce availability of fresh–frozen biopsy material. Great translational efforts, in fact, were devoted at surrogating GEP by flow cytometry or IHC and localizing cellular contributors of TME-based prognostication directly on FFPE material. *In situ* staining of matricellular proteins, such as FN, SPARC, and collagens, as well as IHC or immunofluorescence (IF) quantification of tumor-infiltrating lymphocytes and other immune cells (13–17) provided results partially in line with GEP, but highly controversial due to their low reproducibility and questionable validation. They further underscored that the static pictures of protein or surface marker expression are inadequately representative of the transcriptional dynamism that controls TME components at functional rather than phenotypic level. This aspect is particularly critical for Mo and explains their controversial role in DLBCL prognostication (18). When measured by the sole CD68 IHC staining, the extent of tumor infiltration by Mo appeared significantly associated with an adverse outcome to CHOP therapy only in the study by Cai et al. (19), whereas it had no prognostic value in other studies (13, 20, 21). Conversely, CD68 at both the RNA and the protein levels was found to have a positive prognostic impact in patients treated by rituximab plus CHOP (22). Co-staining of CD68 and CD163—capturing putative immunosuppressive Mo with a M2-like phenotype—correlated with shorter survival in R-CHOP-treated cohorts (23–25), whereas the prevalence of either M1-like $CD68^+$/$HLA\text{-}DR^+$Mo (24) or M2-like $CD163^+$ cells in similar studies did not show any significant prognostic association (22). Such discrepancies not only were mainly due to differences in staining techniques, antibody clones, patient cohorts, and treatments, but also imply that simple detection of surface molecules does not surrogate the extreme *in vivo* functional plasticity of Mo. Recently, a "lymphoma-associated Mo interaction gene" signature (LAMIS) was built

on pooled GEP datasets and associated to shorter PFS and OS in a large cohort of R-CHOP/R-CHOP-like-treated patients, independently of COO and IPI status (26). However, beyond prognostic implications, a fundamental comprehension of Mo biology is still lacking, probably due to insufficient technology to disentangle their quantitative, functional, and phenotypic dynamics within the DLBCL milieu.

On the other hand, as the access to huge amounts of transcriptomic data from bulk tissues became available, the application of new computational tools allowed unprecedented degrees of TME exploration. The deconvolution of GEP or RNA sequencing (RNA-seq) data was shown to provide simultaneous information about quantitative proportions of non-malignant cell types and their transcriptional states, uncovering potential prognostic and therapeutic associations (27–29). In a direct experience of our group, publicly available GEP datasets, including the one by Lenz et al. (8), were analyzed by CIBERSORT (27) to draw maps of the immune/stromal ecosystem in more than 480 R-CHOP-treated DLBCL. Then, the identification of prognostic genes—associated to commonalities between cases in estimated fractions of specific microenvironment cytotypes—represented the first approach exploiting deconvolution to overcome the limits of GEP. Moreover, the prognostic power of the panel was validated by NanoString technology on two independent patient cohorts and demonstrated the feasibility of measuring the expression of TME-related transcripts directly on FFPE diagnostic biopsies (8). An innovative deconvolution framework using CIBERSORTx (29) to combinations of single-cell RNA-seq and bulk transcriptomic data has been very recently reported in *de novo* DLBCL. This approach recognized 49 distinct transcriptional states across 13 main tumor-associated cytotypes, including neutrophils, Mo, fibroblasts, and T cells (30). Patient subsets with peculiar enrichment in TME cell states also showed significant outcome differences that cannot be identified by classical transcriptomics. Consistently, the preliminary results from an independent investigation—applying an alternative algorithm to deconvolve >3,000 DLBCL from 13 transcriptomic and mutational datasets—identified four lymphoma subclasses with distinctive TME traits pairing recurrent genetic drivers of the tumor. Moreover, these new categories show different outcomes, independently of recent molecular classification (31). Such pioneering methods to unify subtle changes in rare TME populations with genetic features of the malignant counterpart provide unprecedented insights in DLBCL biology but require additional effort to prompt their clinical and even therapeutic applicability. **Table 1** summarizes the major published studies exploring DLBCL TME over the last 20 years.

BIOLOGICAL DETERMINANTS OF TME-RELATED PROGNOSTICATION

Taken together, results from both low- and high-resolution dissection of DLBCL outlined aspects of TME dynamics that remained underestimated for years. Molecular signatures reflecting a predominant fibroblastic reaction and Mo infiltration correlated with better outcomes, thus generating a paradoxical interpretation of the common meaning of tumor-associated fibroblasts and Mo (36). Our recent work also emphasizes that biological differences between cases in the validation cohorts may impact on prognosis since they were homogeneously selected based on molecular and clinical parameters [i.e., the validation sets include only advanced-stage patients who have undergone standard front-line R-CHOP/R-CHOP-like regimens (9)].

From a biological point of view, ECM components as well as fibroblasts and Mo appear critically inter-chained as major cross-players of the structural and inflammatory machineries of SLO. Collagens, proteoglycans, glycoproteins, metalloproteinases, and matricellular proteins, such as SPARC and osteopontin, are synthesized by mesenchymal elements and partially by Mo, generating heterogeneous mixtures undergoing continuous remodeling under the pressure of tumor growth and inflammation (37). The deposition of non-cellular factors also mediates the activation of adhesion molecules and integrins (i.e., αVβ3 or α6β4) that provide anchorage to Mo and T cells and possible antigen-independent stimulation of the BCR pathway in malignant cells (38). Paracrine gradients of cytokines and chemokines released by stromal and tumor cells themselves also drive the recruitment and the polarization of monocytes/Mo, T cells, DC, as well as other stromal elements with antigen-presenting capacity, such as follicular dendritic cells and fibroblastic reticular cells (FRC) (39–41). A number of preclinical studies indicated that accessory cells as neutrophils, stromal cells, monocytes, and T cells hold the capacity to modulate tumor survival. Neutrophils can be recruited by CXCL8-secreting tumor cells and, in turn, modulate tumor growth by secreting the proliferation-inducing ligand APRIL and up-regulating the NF-kB, STAT3, and p38 pathways via the Toll-like receptor 9 signal (42–44). Co-cultures of mouse stromal elements with primary DLBCL cells enhanced their clonogenicity as effect of both cell-to-cell adhesion and paracrine mechanisms involving the B cell activating factor and the BCL2 axes (45, 46). Similarly, cells of monocytic origin were proved to prolong lymphoma cell survival by mechanisms that are still unclear (47). All these models, however, remain poorly representative of the *in vivo* complexity of tumor/TME interactions and far from explaining their influence on outcome to standard immunochemotherapy.

An additional influence of TME on lymphoma behavior involves the defective immune competence of effector cells. A PD-L1 overexpression by tumor and TME components is observable in a considerable fraction of DLBCL showing pools of exhausted PD-1$^+$ T cells (48). The phagocytic activity of Mo and DC is likewise hampered by SIRPα stimulation after binding with CD47, which is up-regulated on tumor cells. Both these mechanisms encouraged the experimental use of new anti-PD-1 and anti-CD47 antibodies in relapsed/refractory DLBCL, aiming at restoring the specific immune function of TME (49, 50).

On the other hand, some *in vitro* and *in vivo* results suggest the ability of tumor cells to shape the composition of the surrounding milieu. For instance, genetically unstable DLBCL cells display reduced surface expression of MHC and CD58 molecules, thus lowering T cell and NK infiltration and cytotoxicity (51). Conversely, DLBCL-released lymphotoxins and TNF-alpha were

TABLE 1 | List of studies assessing the prognostic implication of TME in DLBCL.

Technique	Biomarker	TME component	Number of cases/material	Treatment	Prognostic implication	References
IHC/IF	CD1a+ (DC) Granzyme B^+ (T cells)	DC	48/FFPE	CHOP/Rituximab	CD1a+: favorable OS Granzyme B+: favorable OS	(23)
	SPARC CD68	Stromal cells	262/FFPE	R-CHOP/R-CHOP-like	SPARC: favorable OS and FS CD68: not significant	(13)
	SPARC FN1	Stromal cells	173/FFPE	CHOP/CHOP-like R-CHOP/VACOP	SPARC/FN1: favorable OS	(14)
	FOXP3 CD3	T cells	161/FFPE	R-CHOP	FOXP3 and CD3: favorable	(21)
	PD-1 CD3 PD-L1	T cells	414/FFPE	R-CHOP	$CD3^{high}$PD-1+: unfavorable OS PD-1/PD-L1 interaction: unfavorable OS	(32)
	PD-1 LAG-3 TIM-3	T cells	123/FFPE	R-CHOP/other	TIM-3: unfavorable OS and PFS	(33)
	CD68 CD163	Mo	221/FFPE	CHOP/R-CHOP	CD68: unfavorable OS and PFS CD163: unfavorable OS and PFS	(34)
IHC GEP	CD68 CD163	Mo	181/FFPE (IHC) 544/FF (GEP)	R-chemo Chemo	R-chemo: favorable PFS and OS Chemo: unfavorable PFS and OS	(22)
GEP	Lymph node T cell signatures	Monocyte/Mo NK ECM T cells	42/FF	Anthracycline-based regimens	-	(10)
	Host response signature	T cells, monocyte/Mo, DC	176/FF	CHOP	Unfavorable	(7)
	Stromal-1 Stromal-2 signatures	ECM proteins Mo Vascular density	414/FF	CHOP/R-CHOP	Stromal-1: favorable Stromal-2: unfavorable	(8)
	LAMIS signature	Mo	466/FFPE	R-CHOP/R-CHOP like	Unfavorable	(26)
RNA-seq IHC	PD-L1	Mo	702/FFPE (RNAseq) 433/FFPE (IHC)	R-CHOP vs. obinutuzumab-CHOP and R-CHOP±bevacizumab	Favorable	(35)
Deconvolution (CIBERSORT)	45-TME gene panel	Myofibroblasts DC CD4-T cells	482/FF 215/FFPE	R-CHOP/R-CHOP like	Favorable OS and PFS	(9)

reported to promote the proliferative attitude of podoplanin-, PD-L1/L2-positive fibroblasts, while lowering their ability to contract collagen fibers and attract cytotoxic T cells (52).

Overall, it is conceivable that the local extent of constitutional and reactive processes of both stromal and inflammatory nature shapes the final cellular composition of the affected lymph nodes, forming specialized contextures with topographical and functional identity (**Figure 1**). These niches may vary within the same tumor, across different tumor sites in the same patients, and between different patients, resulting in a relevant biological and outcome diversity. The application of innovative computational tools (9, 26) added texture to this picture in DLBCL, yet remaining elusive about the precise mechanisms and timing of TME-centered dynamics. The recognition of a single biological trait unifying the complexity of tumor/TME interactions is very challenging, owing to their potential variation at different disease stages and type of treatment. In fact, the favorable prognostic value observed for stromal/Mo signatures in DLBCL treated by chemo-immunotherapy may rely on the mechanism of rituximab action, which activates killing by the phagocytic capacity of resident immune cells, especially Mo (53). There is indeed growing interest in exploring the role of pure stromal axes, such as SDF-1/CXCR4, in sustaining B cell survival via BCR-independent mechanisms (54) and affecting their sensitiveness to BCR inhibitors (i.e., ibrutinib) and immune modulators (i.e., lenalidomide) with a known off-target effect on both the stromal and the immune components of TME (55, 56).

CONCLUSION AND FUTURE DIRECTIONS

Enormous body of work based on new-generation technologies has produced low/medium-resolution data on the quality of tumor and its surrounding TME, to predict patient responsiveness to standard therapy. While the success of novel immunotherapies increases in other lymphoma subtypes, clinical

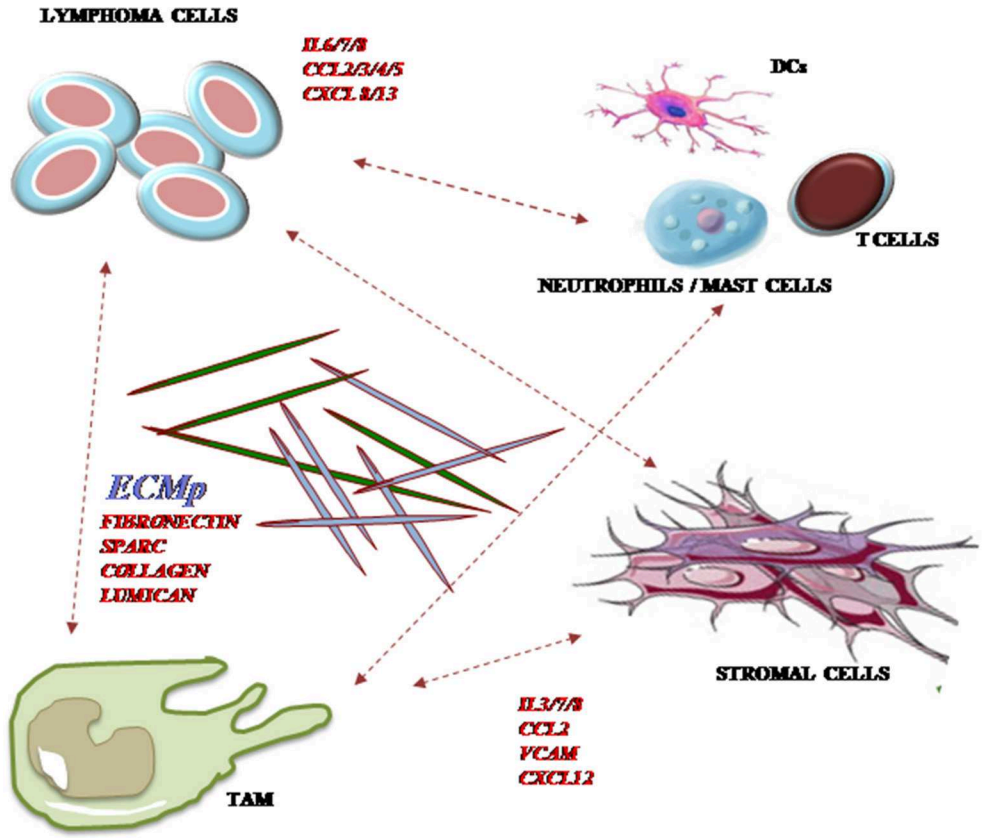

FIGURE 1 | Major cellular and non-cellular components of TME in DLBCL. A number of cellular elements of both immune and stromal origins generate intricate cell-to-cell and paracrine networks with tumor B cells. Reciprocal modulation occurs between malignant clones and tumor-associated macrophages (TAM), stromal and immune cells including neutrophils, mast cells, T cells, and dendritic cells (DC) through the expression of chemokines, cytokines, and extracellular matrix (ECM) component deposition. CCL, CC-motif chemokine ligand; CXCL, CXC-motif chemokine ligand; CXCR, CXC-motif chemokine receptor; ECM, extracellular matrix; IL, interleukin.

results are unsatisfactory in DLBCL. Therefore, characterization of TME is emerging as a critical step for strengthening the rationales of upcoming treatments or enriching subgroups of front-line responder patients. The implementation of computational techniques offers a chance to mine old bulk transcriptomic data and interrogate new sequencing records at a single-cell level. Moreover, the combination of innovative multidimensional applications of digital pathology is expected to provide deeper insights on the composition, function, and localization of immune and stromal determinants of DLBCL.

On the other hand, despite tremendous experimental efforts, it remains of critical importance to clarify (i) whether and how the tumor transcriptional, mutational, and immunogenic landscape influences the TME composition; (ii) how reciprocal stimuli between tumor and immune/stromal cells change as the disease progresses and under the influence of different drugs; and (iii) how the constitutive local feature of the SLO microenvironment influences tumor initiation and progression. Robust preclinical models and *in vivo* ultra-sensitive arrays to measure subtle TME changes will be necessary to answer these questions and translate future results to the clinical setting.

AUTHOR CONTRIBUTIONS

SC and GO discussed, designed, and conceptualized the review article. SC, GO, and MV wrote and edited the article. AG provided the first revision of the article. Literature review was performed by TS, GL, and AN. AN and TS respectively constructed **Figure 1** and **Table 1**. SC and SP performed the final revision of the article.

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Biological Difference Between Epstein–Barr Virus Positive and Negative Post-transplant Lymphoproliferative Disorders and Their Clinical Impact

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Epstein–Barr virus (EBV) infection is correlated with several lymphoproliferative disorders, including Hodgkin disease, Burkitt lymphoma, diffuse large B-cell lymphoma (DLBCL), and post-transplant lymphoproliferative disorder (PTLD). The oncogenic EBV is present in 80% of PTLD. EBV infection influences immune response and has a causative role in the oncogenic transformation of lymphocytes. The development of PTLD is the consequence of an imbalance between immunosurveillance and immunosuppression. Different approaches have been proposed to treat this disorder, including suppression of the EBV viral load, reduction of immune suppression, and malignant clone destruction. In some cases, upfront chemotherapy offers better and durable clinical responses. In this work, we elucidate the clinicopathological and molecular-genetic characteristics of PTLD to clarify the biological differences of EBV(+) and EBV(–) PTLD. Gene expression profiling, next-generation sequencing, and microRNA profiles have recently provided many data that explore PTLD pathogenic mechanisms and identify potential therapeutic targets. This article aims to explore new insights into clinical behavior and pathogenesis of EBV(–)/(+) PTLD with the hope to support future therapeutic studies.

Keywords: post-transplant lymphoproliferative disorders, Epstein–Barr virus, next-generation sequencing, microRNA, gene expression profile, tumor microenvironment

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INTRODUCTION

The World Health Organization (WHO) classification of lymphoid malignancies considers four major diagnostic post-transplant lymphoproliferative disorder (PTLD) categories: early lesions, polymorphic PTLD that could be either polyclonal or monoclonal, Hodgkin lymphoma (HL), and monomorphic PTLD of which diffuse large B-cell lymphoma is most common (1).

PTLD can occur in 20% of hematopoietic stem cell (HSC) and solid organ transplant (SOT) recipients.

PTLD is associated with Epstein–Barr virus (EBV) infection in 60–80% of cases. In EBV infection in immunocompetent (IC) hosts, the virus forms an episome in latently infected B cells (2, 3). In post-transplant patients, immunosuppression causes T-cell inhibition with a consequent lack of T-cell modulation on B-cell proliferation. In particular, when an EBV(–) patient receives an EBV(+) transplant graft, immunosuppression causes uncontrolled proliferation of EBV-transformed B cells, which contributes to the development of PTLD (2, 4, 5).

The pathogenesis of EBV-PTLD is currently unclear; different hypotheses have been suggested as possible pathogenic mechanisms of these EBV-PTLD, such as chronic immune triggering by the graft, hit-and-run EBV infection (EBV induces chromosomal aberrations in cell genome and might be lost during malignant cell division), and other infectious agents [e.g., human herpesvirus 5, 6, or 8; (6–11)]. However, there is limited evidence supporting these hypotheses (**Table 1**).

Clinically, there are differences between EBV(–) and EBV(+) PTLD. In particular, it has been described that EBV(–) PTLD arises later, after years of transplantation, whereas EBV(+) cases arise earlier, generally after months. Furthermore, EBV RNA is detected in early and polymorphic lesions, typical lesions early after transplantation.

In the literature, contradictory data are described regarding the diversity of prognosis between the EBV(+) and EBV(–) cases; in particular, the international multicenter prospective phase 2 PTLD-1 trial found no association with overall survival and EBV status [(22, 23); **Table 2**].

From a therapeutic point of view, EBV(+) and EBV(–) PTLD have the same therapy; the only difference is regarding the EBV-specific adoptive immunotherapy.

Many studies have tried to investigate the genomic differences between the IC-DLBCL, EBV(+), and EBV(–) PTLD. What emerged was that EBV(–) PTLD has a genomic profile very similar to that of IC-DLBCL and a much greater biological complexity than EBV(+) PTLD (26–29).

Furthermore, it has been shown that EBV(+) PTLD, in addition to having a different genomic profile, has different genetic and tumor microenvironment alterations compared with those of EBV(–) PTLD (30–32).

Furthermore, EBV infection may alter the microRNA expression in B lymphocytes. MicroRNA is an important transcriptional and post-transcriptional regulator of gene expression.

In PTLD, EBV(+), B-cell lymphoma revealed different microRNA profiles, compared with normal B cells or EBV lymphoblastoid cell lines generated *in vitro* (33, 34).

These considerations seem to suggest that the pathogenesis of EBV(–) PTLD is to be considered much more similar to that of IC-DLBCL and that it is less influenced by post-transplantation factors. However, despite these differences, the fact that some EBV(–) PTLD respond well to reduction of immunosuppression similarly to EBV(+) PTLD remains to be clarified (35). Certainly, these studies seem to offer theoretical support for future therapeutic studies in EBV(+) and EBV(–) PTLD that appear to have a different pathogenesis.

THE GENOMIC LANDSCAPE OF EPSTEIN–BARR VIRUS POSITIVE AND NEGATIVE POST-TRANSPLANT LYMPHOPROLIFERATIVE DISORDERS

In this work, we want to illustrate the genomic complexity of EBV(+) and EBV(–) PTLD through the integration of different genomic approaches that have significantly improved our understanding of the genetic landscape of these disorders (**Table 3**).

MOLECULAR CHARACTERIZATION THROUGH A GENOMIC APPROACH

Poirel et al. (36) studied PTLD cases with comparative genomic hybridization (CGH) and fluorescence *in situ* hybridization (FISH). The overall incidence of chromosomal imbalances was described in half of PTLD cases, even in the polymorphic category. Latent EBV infection was found in the lesions of three quarters of cases. Non-random losses were 17p13; 1p36, 4q; and 17q23q25, Xp. The gains of 8q24, 3q27, 2p24p25, 5p, 9q22q34, 11, 12q22q24, 14q32, 17q, and 18q21 were the most frequent. Three amplifications −4p16, 9p22p24, and 18q21q23– were detected. FISH has confirmed the involvement of Bcl2 in this latter imbalance. Chromosomal imbalances tended to be more complex in EBV(–) cases than in EBV(+) cases. The identification of chromosomal regions non-randomly involved in lymphomagenesis supports the role of candidate genes to be identified by a combined approach using gene expression profiling (GEP) and CGH array.

In order to improve PTLD pathogenesis understanding, Rinaldi et al. studied recurrent lesions revealed by whole-genome profiling analysis (26). The most common gains in IC-DLBCL were chromosome 3q, 7q, 12, and 18q and in PTLD were chromosomes 5p and 11p. The most common losses in IC-DLBCL were chromosome 12p and in PTLD were 6q, 17p, 1p, and 9p. DNA loss did not always match with loss of heterozygosity (LOH), and uniparental disomy seems to target chromosome 10 in PTLD. They found small deletions and gains involving BCL2 and PAX5 and ZDHHC14 (known gene). These data show that PTLD, at a lower frequency, shares common genetic aspects with IC-DLBCL. 9p13 amplification supports the importance of PAX5 in PTLD pathogenesis. Different DNA copy number and LOH patterns support the hypothesis that uniparental disomy can have a role in lymphomagenesis.

High-density genome-wide single-nucleotide polymorphism (SNP)-based arrays were used by Rinaldi et al. (27) to compare PTLD with IC-DLBCL and to compare EBV(+) with EBV(–) PTLD. In PTLD, the more frequently deleted loci were small interstitial deletions targeting FRA1B, FRA2E, and FRA3B fragile sites. PTLD presents typical and different aberrations than does IC-DLBCL: the deletions at 2p16.1 (FRA2E), lack of del(13q14.3) (MIR15/MIR16), and copy neutral LOH affecting 6p MHC. EBV(+) PTLD presented less recurrent lesions than did EBV(–) PTLD, including a gain of 7p, del(4q25–q35), and gains of 7q, 11q24–q25.

Menter et al. (29) investigated PTLD through next-generation sequencing (NGS) using the Ion Torrent platform. Nuclear factor-κB pathway-related genes had fewer mutations in EBV(+) PTLD compared with IC-DLBCL. Moreover, in PTLD, compared with IC-DLBCL, TP53 was more frequently mutated, whereas ATM and B2M mutations were absent. TP53 mutations were more frequent in EBV(–) PTLD. Mutations in DNA damage control and immune-surveillance genes are different in PTLD

TABLE 1 | Major risk factors in the development of PTLD.

Risk factors for PTLD		
Infectious etiologies	EBV, especially when EBV(–) recipients received a transplant graft from EBV(+) donor. Mismatch for CMV, HCV, and HHV-8, especially when they coincided with EBV infection.	(5, 12)
Age and race	Ages <10 and >60 years. Race: White transplant patients > Blacks.	(13, 14)
Immunosuppressive therapy	The degree, duration, and type of immunosuppression (in particular, anti-thymocyte globulin, calcineurin inhibitors, anti-CD3, tacrolimus, and cyclosporine)	(15, 16)
HSCT/SOT-related factor	SOT types (multi-organ and intestinal transplants have an increasing risk than have lung transplants > heart transplants > liver transplants > pancreatic transplants > kidney transplants). HLA mismatch in HSCT (haploidentical transplants have an increasing risk than have unrelated donor > umbilical cord transplant > HLA-identical related). Type of GVHD prophylaxis, T-cell depletion has the highest risk. Severity of GVHD transplant.	(16–19)
Genetic factors	Polymorphisms in cytokine genes. Recipient HLA, donor polymorphisms.	(20, 21)

EBV, Epstein–Barr virus; CMV, cytomegalovirus; HCV, hepatitis C; HHV, human herpesvirus; HSCT, hematopoietic stem cell transplant; HLA, human leukocyte antigen; GVHD, graft-vs.-host disease; SOT, solid organ transplant.

TABLE 2 | Clinical aspects of EBV(+)/(–) PTLD.

Clinical aspects	**EBV(+)/(–) PTLD**	**References**
Incidence	55–65% of PTLD is associated with EBV infection.	(21, 24)
Clinical presentation	EBV(–) occur later (years) than does EBV(+) PTLD (months). EBV(–) present more often as monomorphic PTLD.	(25)
Prognosis	Controversial results in literature about the different prognoses of EBV(+)/(–) PTLD.	(22)
Therapy and prospective	EBV(+) and EBV(–) PTLD have the same therapy. Specific immunotherapies for EBV(+) PTLD have been proposed, for example, adoptive T-cell transfer, immune checkpoint inhibitors, and antiviral therapy.	(23, 25)

EBV, Epstein–Barr virus; PTLD, post-transplant lymphoproliferative disorder.

with respect to IC-DLBCL. EBV seems to have a role in the different mutational pattern.

MOLECULAR CHARACTERIZATION THROUGH A TRANSCRIPTIONAL APPROACH

Through gene expression analysis, Morscio et al. (38) and Craig et al. (30) showed that EBV(+) and EBV(–) PTLD have different microenvironment and gene expression profiles. They also demonstrated that EBV(–) PTLD and IC-DLBCL are biologically similar.

Through array comparative genome hybridization (aCGH) analysis, Ferreiro et al. (31) studied at genomic and transcriptomic levels EBV(+) PTLD, EBV(–) PTLD, and IC-DLBCL.

EBV(+) PTLD had a different CNA pattern as compared with EBV(–) PTLD and a lower genomic imbalance.

Moreover, EBV(+) PTLD showed distinct aCGH profiles with only one recurrent imbalance with EBV(–) PTLD. On the other hand, EBV(–) PTLD displayed similar recurrent aberrations (gain of 3/3q and 18q and loss of 6q23/TNFAIP3 and 9p21/CDKN2A) as compared with IC-DLBCL. These findings support the concept of a biological relationship between both conditions.

9p24.1 gain/amplification was the most frequent aberration in EBV(+) PTLD targeting PDCD1LG2/PDL2. These genes encode immunomodulatory programmed cell death ligands (39).

In lymphoproliferative disorder, particularly in primary mediastinal B-cell lymphoma, classical HL, and primary central nervous system lymphoma, 9p24.1 is a common copy number gain. The consequence of this alteration is an increase of PDL1 and PDL2 and their induction by *JAK2* (40–43).

An upregulation of PDL1 was described in the majority of EBV(+) lymphomas, including PTLD (44–46). PDL1/2 signal regulates immune defenses against pathogens and T-cell tolerance/T-cell activation through the PD-1 receptor (47).

Green et al. (44) demonstrated an alternative activation mechanism of PDL1 in classical HL and EBV(+) lymphoma, in which EBV latent membrane protein 1 (LMP1) is involved in PDL1 upregulation. These results were also supported by Chen et al. (45), who demonstrated how EBV(+) lymphomas, including PTLD, express detectable PDL1. In lymphomas, genomic amplification or EBV infection causes the PD-1/PDL signaling pathway activation with the immune surveillance escape (**Figure 1**).

TABLE 3 | Genomic characterization of EBV(+) and EBV(–) PTLDs through different technologies approaches.

Genomic approach	EBV(+)/EBV(–) PTLD	References
CGH	The most common copy number aberration in EBV(+) PTLD is the gain/amplification of 9p24,	(36)
FISH	whereas in EBV(–) PTLD, it includes gain of 3/3q and 18q, loss of 6q23/TNFAIP3, and loss of	(26)
WGP	9p21/CDKN2A	(27)
SNP	TP53 mutations were more frequent in EBV(–) PTLD than EBV(+) PTLD and IC-DLBC.	(31)
NGS	Compared with EBV(+) PTLD, EBV(–) PTLD and IC-DLBC have more frequent gene mutations	(29)
	associated with the NF-κB pathway.	(37)
	EBV(+) PTLD has a constitutive activation of the PI3K/Akt/mTOR pathway.	
TRANSCRIPTIONAL APPROACH		
GEP	EBV(–) and EBV(+) PTLD demonstrated different GFP especially gene involved in inflammation	(38)
MicroRNA expression	and immune response pathway profile.	(30)
	EBV(+) PTLD has a suppressed expression of microRNA-194.	(31)
		(33)

CGH, comparative genomic hybridization; FISH, fluorescence in situ hybridization; WGP, whole-genome prediction; SNP, single-nucleotide polymorphism; NGS, next-generation sequencing; IC-DLBC, immunocompetent diffuse large B cell; GEP, gene expression profiling; NF-κB, nuclear factor-κB.

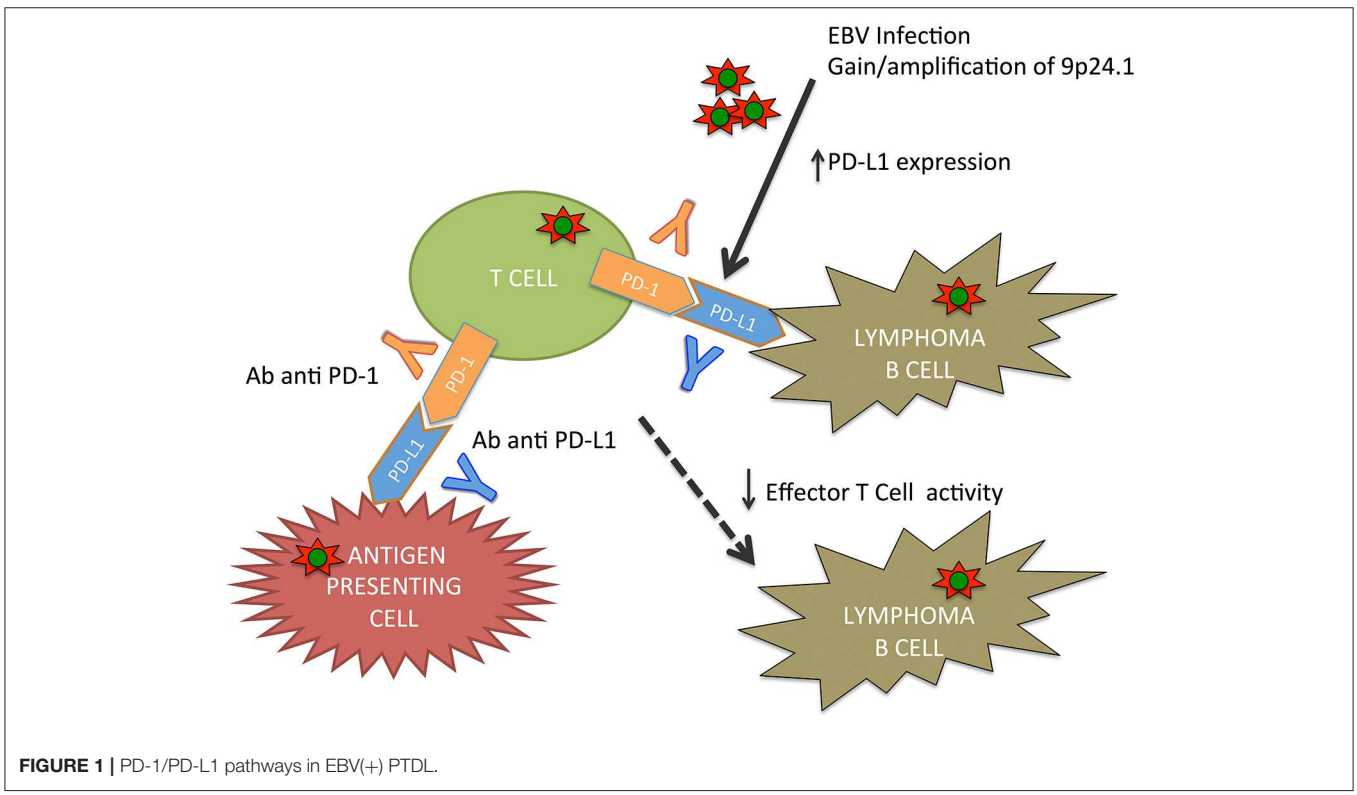

FIGURE 1 | PD-1/PD-L1 pathways in EBV(+) PTDL.

The distinctive copy number alteration in EBV(+) PTLD was identified as a gain of 9p21 with respect to EBV(–) PTLD. Gain of 9p21 caused different CDKN2A expression. CDKN2A codes for cyclin-dependent kinase inhibitor 2A (p16INKA), an important regulator of the cell cycle; in particular, it decelerated cell progression through the G1 phase (48). In EBV(+) PTLD, immunohistochemistry (IHC) demonstrated that a gain of 9p21 was associated with exclusively cytoplasmic expression of the p16INKA protein. The p16INKA seems to be implicated in alternative oncogenic pathways and not as a tumor suppressor in EBV(+) PTLD (48, 49).

A gain of chromosome 3/3q was found in EBV(–) PTLD, and it was absent in EBV(+) PTLD. This alteration caused increased expression of FOXP1 in EBV(–) PTLD; these data were confirmed by QRT-PCR and IHC. FOXP1 encodes a transcriptional regulator implicated in different biological processes and in B-cell lymphomas pathogenesis; and it seems to play a critical role in the pathogenesis of EBV(–) PTLD. However, the connection between EBV infection and FOXP1 is uncertain because EBV downregulates FOXP1 in normal B cells (50–54).

IC-DLBCL has many points in common with EBV(–) PTLD. EBV(–) and EBV(+) PTLD demonstrated different genomic

and gene expression profiles. In particular, GEP differences in EBV(+) and EBV(–) PTLD involve inflammation and immune response pathways (31), supporting the hypothesis that the EBV infection has a major impact on the gene expression and alterations in EBV(+) PTLD. On the other hand, the EBV(–) PTLD appears to be more similar to *de novo* lymphomas arising in transplanted patients.

Many studies support the role of cytokines in the pathogenesis of EBV(+) PTLD (55). This hypothesis is supported by the detection of IL-10 transcripts in PTLD biopsies. B-cell lymphomas isolated from EBV(+) PTLD produce IL-10 in a constitutive way and use it as an autocrine growth factor (56). For this reason, serum IL-10 has been proposed as an early marker of PTLD (57–60). It is unclear why IL-10 is altered in EBV(+) PTLD.

EBV infection modifies microRNA expression. Gene arrays demonstrate different microRNA profiles in EBV(+) B-cell lymphoma lines from patients with PTLD, as compared with *in vitro* generated EBV(+) lymphoblastoid cell lines or normal B cells. In particular, microRNA-194 (33) was found to be suppressed in EBV(+) PTLD. MicroRNA-194 overexpression increases apoptosis of EBV(+) B-cell lymphoma lines and attenuates IL-10 production. EBV seems to suppress microRNA-194 in order to increase IL-10 expression. Therefore, microRNA-194 may constitute a new approach to inhibiting proliferation of EBV(+) B-cell lymphomas in PTLD.

CONCLUSIONS AND PROSPECTIVE

In recent years, increasing understanding of the biologic and molecular PTLD pathogenesis has resulted in new therapeutic approaches and improved outcomes for these patients. Although the prognosis of EBV(+) in comparison with EBV(–) PTLD is not clear, frontline therapy in EBV(+) and EBV(–) PTLD is currently the same.

In this work, we reported much evidence that EBV(+) and EBV(–) PTLD have distinct genomic and transcriptomic landscape, although at the moment, clinical data do not completely support this hypothesis. EBV(–) PTLD and IC-DLBCL seem to be similar biological entities; for this reason, EBV(–) PTLD might be considered as a type of lymphoma that develops coincidentally in transplant recipients. Moreover, EBV(+) PTLD and EBV(+) DLBCL present many similarities, indicating that EBV both infection and reactivation have important consequence on their pathogenesis (30–32, 38).

PTLD therapy is a combination of reduction of immunosuppressive therapy, immunotherapy, and chemotherapy (23, 25). In this review, we summarize the clinical and biological differences of EBV(+) and EBV(–) PTLD, and we support a new therapeutic approach based on EBV status to improve outcomes of these patients.

The expression of viral antigens makes EBV(+) PTLD an attractive candidate for specific therapy. Unfortunately, latent EBV-infected B cells do not express EBV-thymidine kinase transcript/protein; and for this reason, they are unaffected by antiviral agents as purine nucleoside analog. Similarly, EBV-related lymphoproliferative disorders do not express viral protein kinase, and so monotherapy with nucleoside analogs failed to induce responses in EBV(+) PTLD. However, pharmacological induction of viral thymidine kinase by the administration of the histone deacetylase inhibitor arginine butyrate, followed by antiviral therapy, has shown promising results with an acceptable toxicity profile (61).

More recently, several studies demonstrated how immunomodulatory drugs such as lenalidomide or proteasome inhibitors, in particular bortezomib, can induce EBV lytic activation (62, 63).

The search for new antivirals is ongoing; in particular, a new antiviral agent hexadecyloxypropyl-cidofovir (HDP-CDV) exhibits a remarkable increase in antiviral activity *in vitro* against different double-stranded DNA viruses including EBV (64).

Constitutive activation of the PI3K/Akt/mTOR pathway was shown in *in vitro* EBV(+) PTLD cell lines. Inhibition of either Akt or PI3K, with specific inhibitors CAL-101 or MK-2206, respectively, suppresses EBV(+) PTLD cell growth; and the combination of rapamycin had a synergistic effect. The combination therapy with an Akt inhibitor, or a PI3K inhibitor, and rapamycin can be an efficacious treatment for EBV(+) PTLD (37).

Most results presented are based on *in vitro* data; further evaluation and prospective clinical trials are necessary before such agents can be used as a treatment for PTLD patients.

The upfront treatment of EBV(+) and EBV(–) PTLD is the same, except for the use of EBV-specific adoptive immunotherapy. Immune-based therapies are an effective approach because of EBV antigen expression. In particular, adoptive therapy is based on the high efficacy of unselected donor lymphocyte infusions in HSC transplantation PTLD (65). Attempts were made to isolate EBV-specific cytotoxic lymphocytes (CTLs) aiming to induce a strong EBV-specific cellular immune response without the risk of graft-vs.-host disease (GVHD). Both autologous and allogeneic [isolated from the donor itself or a partial human leukocyte antigen (HLA)-matched donor] CTLs, targeting specific immunogenic EBV antigens, can be used (66). In a large multicentric study, HSCT patients were treated with EBV-CTLs, either prophylactically or therapeutically (67). A Chinese prospective study in HSCT recipients demonstrated an increase in complete remission rates in patients treated with sequential administration of rituximab and EBV-CTLs (68).

Moreover, checkpoint inhibition seems to be a potential treatment option in EBV(+) PTLD. EBV infection/reactivation causes a cytotoxic T-cell dysfunction in lymphomas as PTLD and classical HL. EBV causes an upregulation of immune checkpoint markers. In classical HL, immune checkpoint inhibitors have demonstrated efficacy; and therefore, there has been an increasing interest in PTLD (69). Antigen-presenting cells express PD-L1 that bind the PD-1 receptor on T cells, thus inhibiting T-cell receptor functions. EBV plays a role in increasing PD-L1; these data support the role of checkpoint inhibition in PTLD (44). Kinch et al. demonstrated than PDL-1, PDL-2, and PD-1 were positive in more than half of PTLD cases

following SOT (70). More clinical data are necessary to determine the safety, efficacy, and graft rejection risk or GVHD of immune checkpoint inhibitors in PTLD. Currently, a phase II trial (NCT03258567) of nivolumab in a cohort of patients—EBV(+) non-HLs including EBV(+) PTLD—is ongoing.

This review summarizes many steps that have been made in understanding the EBV(+)/(–) PTLD biology. The biological differences connected with the EBV status support the development of preventive/preventive strategies against EBV disease and implementation of existing therapies both in the frontline and in the setting of relapsed/refractory patients. Several molecular targeting agents including immunomodulatory agents, proteasome inhibitors, PI3K and Akt inhibitors, novel anti-CD20 monoclonal antibodies, and immune checkpoint inhibitors seem to have a therapeutic potential, providing a strong rationale for new clinical trials to improve the outcome of EBV post-transplant lymphoproliferative disorder.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Actionable Strategies to Target Multiple Myeloma Plasma Cell Resistance/Resilience to Stress: Insights From "Omics" Research

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While the modern therapeutic armamentarium to treat multiple myeloma (MM) patients allows a longer control of the disease, this second-most-frequent hematologic cancer is still uncurable in the vast majority of cases. Since MM plasma cells are subjected to various types of chronic cellular stress and the integrity of specific stress-coping pathways is essential to ensure MM cell survival, not surprisingly the most efficacious anti-MM therapy are those that make use of proteasome inhibitors and/or immunomodulatory drugs, which target the biochemical mechanisms of stress management. Based on this notion, the recently realized discoveries on MM pathobiology through high-throughput techniques (genomic, transcriptomic, and other "omics"), in order for them to be clinically useful, should be elaborated to identify novel vulnerabilities in this disease. This groundwork of information will likely allow the design of novel therapies against targetable molecules/pathways, in an unprecedented opportunity to change the management of MM according to the principle of "precision medicine." In this review, we will discuss some examples of therapeutically actionable molecules and pathways related to the regulation of cellular fitness and stress resistance in MM.

Keywords: proteotoxic stress response, autophagy, replication stress, therapeutic targets, Omics analyses

INTRODUCTION

Multiple myeloma (MM) is the most frequent neoplastic disorder affecting post-germinal center B cells and plasma cells, the final stage of B-lymphocyte differentiation (1–3). Despite the clinical severity and dismal prognosis that still characterize MM, the overall survival of affected patients has consistently improved over the last two decades (the 5-year survival rate has nearly doubled) thanks to the application of autologous stem cell transplantation, the use of novel agents and the introduction of maintenance therapy (2). New drugs that have substantially revolutionized the anti-MM therapies are proteasome inhibitors, immunomodulatory agents, and the anti CD38 and anti-SLAMF7 monoclonal antibodies directed against specific plasma cell surface molecules.

More recently, a great deal of research efforts are being devoted to the immunotherapy with anti-B-cell maturation antigen (BCMA) Chimeric Antigen Receptor (CAR)-T cells or bispecific T cell engagers (2, 3). Nonetheless, MM remains a difficult-to-eradicate tumor because it displays a great predisposition toward biological heterogeneity and clonal evolution in time and space that ultimately confers resilience to stress and resistance to cytotoxic agents (4–8). Being the pathobiological features of MM as such, the identification of targets that sustain MM cell "invulnerability" seems a central research goal to pursue.

In this review, we have examined some facets emerging from the body of high-throughput data of functional genomics, transcriptomics, gene silencing, and drug screen that deal with potential vulnerable targets of MM biology liable to therapeutic targeting. We will first discuss the pathways active in MM involved in the management of the proteotoxic/autophagic stress and the replicative/oxidative stress and then analyze the available data coming from -OMICS and functional screens that may allow to design novel therapeutic approaches targeted against stress-managing mechanisms.

CHRONIC STRESS AND PATHWAYS OF STRESS MANAGEMENT IN MM

Endoplasmic Reticulum (ER) Stress/Unfolded Protein Response (UPR) and Autophagy

The protein overload to which MM cells are subjected (due to their activity as antibody producing/secreting cells), is cause of a massive chronic proteotoxic stress, which needs to be managed (9–11). The ER is a chief organelle of accumulation of aberrant proteins and three main homeostatic stress-managing pathways are activated to avoid the potential damage from these misfolded proteins. The UPR-related, ER-resident stress sensors IRE1α, PERK, and ATF6 are activated by the accumulation of misfolded proteins (12, 13). Each of the sensors triggers a signaling cascade that leads to changes in the expression levels of chaperones and other enzymes in order to assist protein maturation or degradation (14) (**Figure 1A**). Moreover, autophagy, which is also essential for normal and malignant plasma cell development, may compensate in part for an impaired UPR/proteasome response by assisting the resolution of proteotoxic stress through the recycle of proteins and organelles and avoiding cell death (15, 16). In particular, autophagy conveys cellular components to lysosomes, through the formation of autophagosomes and autolysosomes with the activation of a series of autophagy related proteins (ATGs) such as ATG7, ATG8, ATG12, ATG5, ATG10, and the conjunction of LC3-I with phosphatidylethanolamine (PE) to form the lipidated form LC3-II (17) (**Figure 1B**). A functional node is represented by the proteasome, the cellular machinery in charge of the proteolysis of polypeptides, which warrants a correct protein turnover (18). However, an overwhelming or prolonged proteostatic/proteotoxic stress represents and Achilles's Heel that may eventually elicit apoptosis (14).

Replication and Oxidative Stress

Recent evidence has highlighted the importance of the replication and oxidative stress in MM (19–21). Targeting the addiction to proliferating cell nuclear antigen (PCNA), which is overexpressed in MM and is essential for replication and DNA damage response, caused MM cell apoptosis, and growth arrest, while no effects were observed in non-malignant cells (19). It was also demonstrated that, due to the strong replicative pressure to which MM cells are subjected, a chronic activation of the DNA damage response occurs in MM cells (20). In these processes, the oncogene MYC plays a central role. Again, the addiction that malignant plasma cells develop to the replication and oxidative stress-managing pathways, i.e., to the ATR and SOD enzymes, accounts for the strong anti-proliferative and pro-apoptotic effect obtained by the inhibition of these two proteins (20). Similarly, MM cells are addicted to RECQ1 helicase, an enzyme involved in DNA unwinding and maintenance of chromosome integrity. RECQ1 is overexpressed in MM and its relationship with an enhanced resistance to replicative stress, could confer in turn a higher resilience of malignant plasma cells toward the cytotoxic effects of chemotherapy (22). Therefore, targeting this protein has been suggested as a potential strategy to increase MM cells susceptibility to replication stress and apoptosis (21).

Oxidative stress, a hallmark of cancer, has also been demonstrated to play a major role in MM. Malignant plasma cell intrinsic generation of reactive oxygen species (ROS) may emanate from genotoxic stress, replicative stress, and proteotoxic stress (5). Several studies that have investigated the expression of various regulators have demonstrated a status of oxidative stress in MM cells and blood samples from patients (23–26). It has also been shown that oxidative stress is modified upon conventional and novel anti-myeloma agents (27, 28). Moreover, expression/modulation of oxidative stress pathway components may influence the responsiveness of MM cells to certain cytotoxic agents (29–31). Recently, it was demonstrated that the cytotoxicity on MM cells of melphalan, a central chemotherapeutic in MM treatment, is partly mediated by the generation of oxidative stress and can be antagonized by antioxidant mechanisms. Glutathione, a physiological anti-oxidant agent, could reduce melphalan-induced apoptosis and cell cycle alterations but this effect was independent from melphalan-induced DNA damage (32).

GENETICS AND GENOMICS OF MULTIPLE MYELOMA AND PATHWAYS OF STRESS MANAGEMENT

Insights into the genomic landscape of MM came from a number of important studies that have investigated the disease's genome through whole exome or whole genome sequencing. In the paper by Chapman et al. (33), a first global analysis of malignant plasma cells from 38 newly diagnosed MM (NDMM) patients has been conducted. This report highlighted the most frequent mutations in NDMM by analysis of whole genome sequencing (WGS) of 23 cases and of whole exome sequencing (WES) of 16 cases (being one patient sample subjected to both

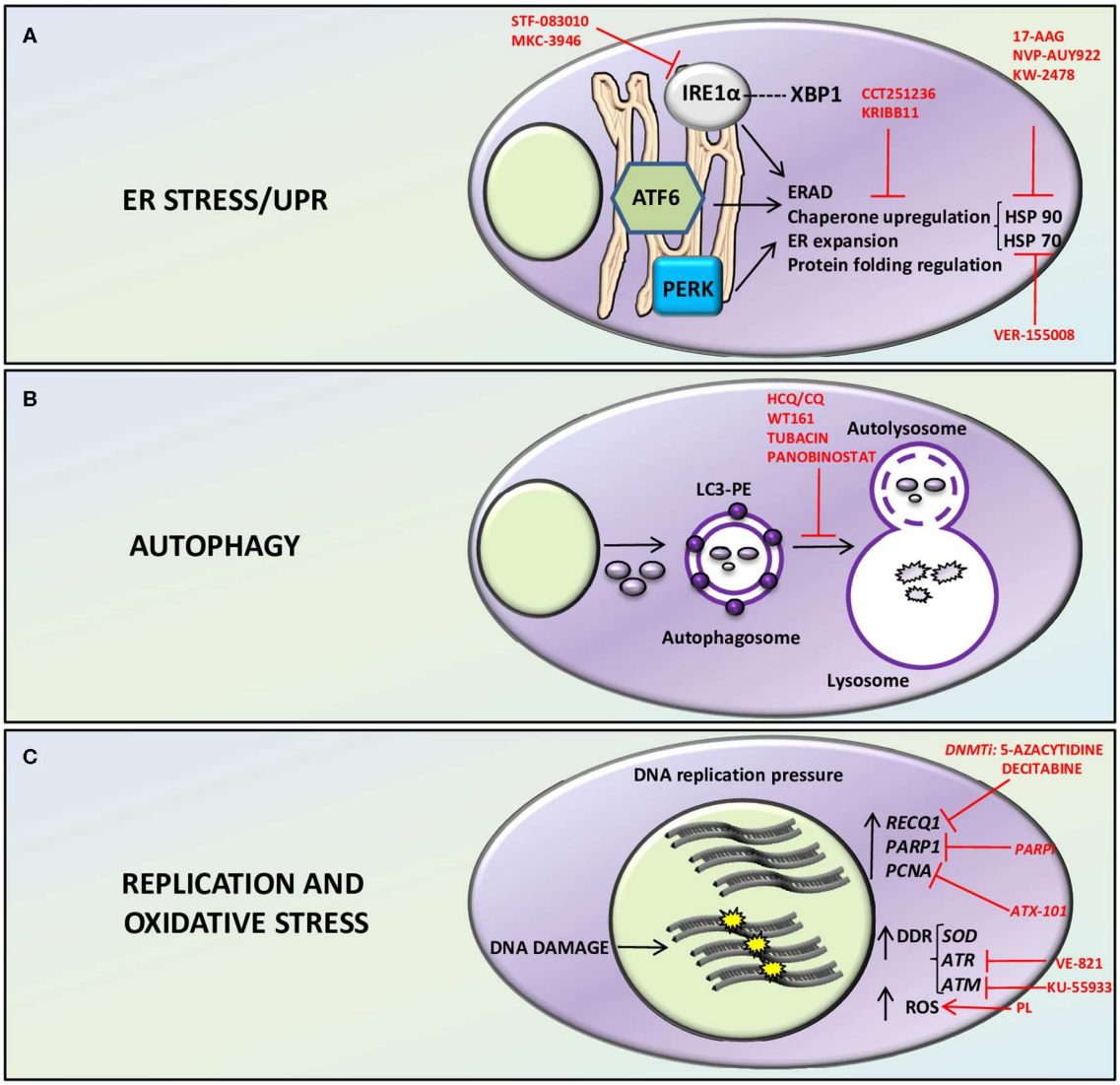

FIGURE 1 | Therapeutically relevant Stress related pathways in MM. ER stress/UPR **(A)**, autophagy **(B)**, and replication and oxidative stress **(C)** related pathways and putative therapeutic targets in MM plasma cells. **(A)** the ER, resident stress sensors IRE1α, PERK, and ATF6 are activated by accumulation of misfolded proteins and sustain MM cell survival by ERAD (ER associated protein degradation) and by protein folding regulation through upregulation of chaperones such as HSP70 and HSP90. Inhibition of HSP90 with 17-AAG, NVP-AUY922, KW-2478, or HSP70 with VER-155008 or both chaperones via inhibition of their regulator HSF-1 with the compounds CCT251236 or KRIBB11 has cytotoxic effect on MM cells. IRE1α inhibition with STF-083010 or MKC-3946 displayed anti-myeloma activity. **(B)** Autophagy, by recycling proteins and organelles avoids cell death. In particular, it conveys cellular components to lysosomes, through the formation of autophagosomes and autolysosomes with the activation of a series of autophagy related proteins (ATGs) and the conjunction of LC3-I with phosphatidylethanolamine (PE) to form the lipidated form LC3-II. Inhibitors of autophagy such as Hydroxychloroquine (HCQ)/Chloroquine (CQ), or HDAC inhibitors such as WT161, tubacin, and panobinostat has been employed to interfere with autophagy and hamper protein homeostasis, leading to MM cell death. **(C)** MM plasma cells display high DNA replication pressure with the consequent likelihood of exposition to DNA damage. MM plasma cells rely on high expression of replication related proteins such as PCNA, PARP, and RECQ1 helicase. DNA damage response (DDR) and oxidative stress are chronically activated in MM through the stimulation of DNA stress managing proteins such as ATR, ATM, SOD, and ROS production. RECQ1, PARP1, PCNA, and DDR inhibitors and compounds that interfere with oxidative stress are depicted in the figure. DNMTi, DNA methyltransferase inhibitors; PARPi, PARP inhibitors; PL, Piperlongumine.

experimental techniques). Confirming previous reports, *KRAS* and *NRAS* were the most frequently mutated genes found (overall in 50% of cases), followed by *TP53* (8%). To note, the clonal drift from *KRAS* to *NRAS* may confer a worse prognosis (34). Newly described mutations to *CCND1* (CyclinD1) gene were also detected in 5% of cases. One extremely interesting finding of this work was the frequent incidence of mutations affecting genes involved in cellular processes deeply connected with cellular stress management (such as RNA processing, protein translation, and the unfolded protein response) in roughly 50% of patients. The most frequently mutated genes were *DIS3/RRP44* in 11%, *FAM46C* in 13%, *LRRK2* in 8% of cases. Also, mutations in

the gene coding for the transcription factor XBP1 (described above as instrumental for plasma cell development and function and ER stress response), have been recognized in 5% of cases. Altogether, 42% of cases were found to have mutations affecting protein homeostasis. Another interesting result was the finding of an accumulation of mutations to members of pathways involved in chromatin regulation, NF-κB transcription factor, and coagulation cascades. Eleven NF-κB pathway genes were found mutated: *BTRC*, *CARD11*, *CYLD*, *IKBIP*, *IKBKB*, *MAP3K1*, *MAP3K14*, *RIPK4*, *TLR4*, *TNFRSF1A*, and *TRAF3*. Also, the discovery of an impaired H3K27Me3 in the *HOXA9* gene with consequent aberrant upregulation of the expression of this transcription factor could be ascribed to mutations affecting histone methylation regulators *MLL*, *MLL2*, *MLL3*, *UTX*, *WHSC1*, *WHSC1L1*. These mutations are of pathogenetic importance in a subset of MM cases, since it was demonstrated that *HOXA9* overexpression may confer a growth advantage to MM cells (35).

Clinically actionable mutations to *BRAF* were also discovered in this first report, which analyzed many MM patient samples. The *BRAF* G469A mutation in one of the 38 patients and the *BRAF* K601N and *BRAF* V600E mutations in 4% of additionally sequenced 161 cases, point to a pathogenetic role of the BRAF regulated signaling, which could be targeted by BRAF inhibitors (36).

Subsequently, a refined analysis including copy number alterations that was powered (<30x sequence coverage) for detecting clonal heterogeneity, has been performed. A larger set ($n = 203$) of NDMM and treated MM patients' samples was examined, 177 by WES, and 26 by WGS (37). Eleven recurrently mutated genes were identified, some of which known (*NRAS*, *KRAS*, *TP53*, *FAM46C*, *DIS3*). Other genes were confirmed mutated (*BRAF*, *PRDM1*, *RB1*, *TRAF3*, *CYLD1*), which are known/believed genes to be of pathogenic importance in MM. The data from the earlier study relative to the pathway-level mutations were confirmed with regard to the NF-κB, coagulation cascades, and histone methylation pathways (37). Analysis of clonal (likely earlier) versus subclonal (later) mutations revealed that driver mutations are not always clonal. For instance, KRAS mutations were detected in 73% of cases as clonal and 27% of cases as subclonal events. Rarely, *KRAS*, *NRAS*, and *BRAF* mutations were found both in the same clone, while this was true for the *DIS3* and *KRAS* mutations. This finding has implications for targeted therapy. Indeed, analyzing *BRAF* mutations as actionable targets, it was demonstrated that while *BRAF*-mutated MM cells are sensitive to BRAF inhibition, a paradoxical growth-promoting effect of BRAF inhibitors is present in MM cells with WT *BRAF*, due to a hyper-activation of the MEK/MAPK pathway (37).

Another approach was used in a mix confirmation/discovery study in NDMM, in which it was confirmed that in MM there are commonly mutated genes and a long tail of uncommonly mutated genes (38). The NF-κB pathway and the DNA damage (*TP53*, *ATM*, *ATR*, *BRCA2*) response pathways were confirmed and identified as recurrently mutated. To note, when the data were interrogated in the context of proteasome inhibitor treatment, no correlations could be found with any alterations, perhaps due to the relatively small sample size (38).

Walker et al., through the use of integrated genomics investigated the mutational landscape, copy number variations, primary translocations and hyperdiploid status in a large cohort of 1,273 newly diagnosed MM patients (derived from the Myeloma XI trial, the Dana-Faber Cancer Institute/Intergroupe Francophone du Myelome, and the Multiple Myeloma Research Foundation CoMMpass study), finding 63 MM driver genes. Among them, some were already previously known (such as *FGFR3*, *DIS3*, *FAM46C*, *MAF*, *BRAF*, *MYC*, *CCND1*, *ATM*, *IRF4*, *PRKD2*, *NF-KB* signaling pathways related genes), some others were new, (such as *IDH1*, *IDH2*, *HUWE1*, *PTPN11*, *KLHL6)* (39).

Bolli et al. (40) have also characterized the genomic landscape of 11 smoldering MM (SMM) by WGS. This analysis has detected on average of 5,308 mutations and 4,397 small *indels* per patient. Important findings included the frequent MYC translocation with non-immunoglobulin heavy (IgH) chain locus partner (5/11) and the overall pattern of driver alterations similar to overt MM, indicating a clear earliness of their onset during myelomagenesis (40). Analysis of a significant interaction between driver events revealed two associations, between *PRDM1* deletions and *t*(4, 14), which confers a worse OS and between *PRDM1* deletions and *BIRC2/3* deletions, which confers a better OS.

Maura et al., through WGS data of 67 MM genomes from 30 patients collected at different times, in association with whole exome data from 804 patients within the CoMMpass trial (NCT01454297) deeply delineated MM genomic subgroups, taking into consideration the mutational landscape, copy number variation, and structural variants. The authors identified 55 distinct genes altered, and among others, they revealed novel driver mutations in *ABCF*, *ZFP36L1*, *TET2*, *ARID2*, *KDM6A*, and *EP300* genes and in the linker histones HIST1H1B, HIST1H1D, HIST1H1E, and HIST1H2BK. They next chronologically reconstructed in a comprehensive manner, driver events in MM pathogenesis (41).

Vikova et al. (42) analyzed through WES, the molecular signature of 30 MM cell lines and 59 primary MM tumors, comparing with eight control samples revealing different mutated driver genes and pathways associated to drug resistance. Novel mutated genes were linked to mitosis, DNA repair processes, chromatin remodeling, and epigenetic modifiers, (such as *CNOT3*, *KMT2D*, *SETD2*, *MSH3*, *PMS1*, *EZH2*), protein trafficking (such as USP6) and altered signaling cascades were associated to the PI3K/AKT (mutations in *TSC1*, *TSC2*, *TBX3*, *PTEN*, *IKBKB* genes), MAP kinase (*MAP2K2*, *RAC1*, *RAF1*, *NF1* mutated genes), JAK/STAT (*STAT3*, *RUNX1*, *EPAS1*, *JAK2*, *STAT6* mutated genes) P53/cell cycle (*TP53*, *ATM*, *CCND1*, *RB1*, *CDKN2A*) some of which are potential targets from the therapeutic point of view. *KMT2D and SETD2* were mutated only in patients at relapse. Moreover, some mutated genes were associated to drug resistance, such as *FAM46C* and *KRAS* (panobinostat), *KMT2D* (dexamethasone), *PMS1* (TSA), and USP6 (SAHA). *KMD2* mutations were also related to lenalidomide sensitivity.

Tessoulin et al. (43) through WES of human MM cell lines found driver genes related to chromatin regulation/modification and DNA repair, associated to drug resistance.

Altogether, these OMICS data suggest that targeting stress associated pathways such as DNA damage response or epigenetic modifiers could offer therapeutic alternatives in MM.

TRANSCRIPTOMICS OF MM AND PATHWAYS OF STRESS MANAGEMENT IN MM

Transcriptomic analysis has been applied to MGUS, SMM, and MM and has identified associated gene expression signatures (44). Gene expression studies have also led to the recognition of the cyclin D overexpression signature as a common feature of MM (45) and of the chromosome 1 transcriptional deregulations as prognostic alterations in high risk MM (46).

Molecules mis-expressed in MM belong to different pathways and functions, including stress-managing pathways, UPR/ER stress, proteasome, and mitochondria function. Recently, the work of Jang et al. (47) was able to dissect the spatial and temporal heterogeneity of MM plasma cellular clones using scRNA-Seq expression profiling at the single cell level in 15 plasma cell dyscrasia patients that included 3 MGUS, 4 SMM, 5 NDMM, and 3 relapsed/refractory MM (RRMM) analyzing a total of 597 cells. The authors identified subpopulations of cells within the same patient sample that express different levels of the same gene, accounting for the heterogeneity expression profiling of different plasma cells within a patient. Cells were clustered into four subpopulations (L1–L4) according to gene expression, being cells in L1 group identified by the lowest expression of genes involved in oxidative stress, MYC target, and mTORC1 dependent signaling pathway. Interestingly, the expression profiles within the four groups correlated with disease progression, most of the cells belonging to MGUS patients clustering in the L1 subgroup. Indeed, this subgroup showed the lowest expression of genes linked to cell metabolism and protein homeostasis, such as all the 18 genes coding for the proteasome subunits, UPR related genes, and genes associated to mitochondria metabolism and function. The authors identified a signature of 44 genes that are consistently related to MM progression. Among these, 26/44 (59%) were linked to UPR/ER stress (such as *ARF1*, *ATF6*, *EIF2a*, *ERLEC1*, *CD46*, *BSCL2*, *CDK2AP2*, *IER3IP1*, *IFNAR1*, *PSMB1*, *SLAMF1*, *SSR2)* and mitochondria (such as *ATP5G1*, *ATP5J*, *DAP3*, *GNG5*, *JTB*, *ROMO1*), underlying the importance of stress managing genes expression in the disease progression (47). Of note, kinases such as *CSNK1A1* and *CSNK2B*, which were shown to be essential for MM plasma cell survival and proteotoxic stress handling (48–50), have been found altered within the four groups, with increasing expression from L1 toward L4. Also, autophagic gene expression (such as *ATG3*) has been found altered among the groups. ATG3, is important for LC3 lipidation, and therefore is essential for autophagocytosis.

Heat shock proteins are essential chaperones that ensure the correct protein homeostasis and folding and helped the management of MM stress, due to hyperactivity of the protein machinery in the antibodies secreting malignant plasma cells. It has been shown that chaperone genes such as *HSPA9* and *HSPE1* coding respectively for GRP95/HSP70 and HSP10 are significantly differentially (higher) expressed in L2–L3–L4 groups compared to L1. Moreover, increased expression of protein homeostasis related genes in plasma cells in the L2–L3–L4 groups was linked to disease progression and reduced OS of MM patients.

Liu et al. (51) analyzed the gene expression profile, copy number variation, and clinical features in a large data set from the Multiple Myeloma Research Consortium (MMRC) identifying eight prognostic signatures encompassing 178 genes related to cell cycle progression and a molecular gene signature involved in immunomodulatory drugs and proteasome inhibitors response. The authors were able to create a MM molecular causal network model, by integrating gene expression and copy number variation data, with supposed key regulators, such as genes involved in cell cycle and metabolic pathways. The results not only identified genes already known to be altered in MM, such as translocations occurring between the heavy chain of immunoglobulins and known oncogenes (*CCND1*, *CCND3*, *MAF*, *FGFR3*, *MMSET*), but also two novel nodes composed by *Alkylglycerone Phosphate Synthase (AGPS*) and *Alpha Thalassemia/Mental Retardation Syndrome*, *X-Linked* (*ATRX*), which regulate multiple genes (41 and 32, respectively). The *AGPS* gene is involved in lipid biosynthesis, a process that many have shown to play a fundamental role in MM progression. Targeting AGPS could therefore be of potential benefit to increase MM cell death, since multiple AGPS inhibitors are under development in other cancers (52).

The *ATRX* gene has been involved in chromatin remodeling, and could be also therapeutically targetable in MM. It has been previously shown that it is a mutational driver (39). Moreover, altered genes were found in molecular pathways related to cell cycle, mitosis, macromolecule biosynthesis, DNA damage response such as *NOP16*, *CECR5*, *MELK* and *TPX2*, *NCAPG2*, *CDK1*, and *DTL*, for many of which there are already inhibitors available that could trigger MM cell apoptosis and cell cycle arrest.

Similarly, the authors identified a treatment response signature which is characterized by the deregulation of genes involved in protein folding and trafficking, such as *FKB5* and the HSP70 cochaperone *DNAJA1*, which could also be therapeutically relevant.

Altogether, once more, these results highlight the importance of the potential targeting of stress managing genes to increase plasma cell vulnerability, implementing MM therapy efficacy.

Table 1 shows a list of MM-related genes found altered in pathways essential for plasma cell dyscrasias through OMICS research.

OTHER OMICS: RNA$_I$, CRISPR/CAS9, AND DRUG FUNCTIONAL SCREENING IN MM

Other approaches have been employed in the search of new therapeutic targets in MM and to overcome drug resistance. Targeted transcriptome/genome editing (RNAi or CRISPR/CAS 9) or high- throughput cell-based drug screening have been

TABLE 1 | List of MM related pathways genes found altered in plasma cell dyscrasias through "OMICS" research.

Altered MM pathways	Genomics or transcriptomics
NF-κB signaling	BTRC, CARD11, IKBIP, IKBKB, MAP3K1, MAP3K14, RIPK4, TLR4, TNFRSF1A, (33) TRAF3, CYLD (33, 37, 39, 41, 43), TRAF2, NFKB1, NFKB2 (39), TNFAIP3, *CD74*, BIRC2, *BIRC3*, *IL2R4*, *NFE2L3* (43).
MAPK signaling	*BRAF* (36, 37, 39, 42), MAP2K2, RAC1, RAF1, NF1 (42).
PI3K signaling	TSC1, TSC2, TBX3, PTEN, IKBKB (42).
JAK/STAT signaling	STAT3, RUNX1, JAK3, STAT6, EPAS1 (42).
GTP ases	*KRAS*, *NRAS* (33, 37, 41–43).
ER stress/UPR/trafficking	*XBP1* (33), *ARF1*, *ATF6*, *EIF2a*, *ERLEC1*, *CD46*, *BSCL2*, *CDK2AP2*, *IER3IP1*, *IFNAR1*, *PSMB1*, *SLAMF1*, *SSR2*, *HSPA9*, *HSPE1* (47), *FKB5*, *DNAJA1* (51), *ABCF1* (41), USP6 (42).
Apoptosis/transcriptional regulators	*MAFB*, *MYC*, *MAX*, *HUWE1* (39), *MAF* (39, 41), BIRC2, *BIRC3*, *EGR1*, *LP1*, *BCL2L11*, *BIM* (43).
Autophagy	*LLRK2* (33), *ATG3* (47).
Replicative stress/DNA repair	*TP53* (33, 37–39, 42, 43) *CCND1* (33, 39, 41, 42) *H3K27Me3 in HOXA9* (33), RB1 (37, 42), ATM, ATR, BRCA2 (38, 40, 43), ATM, MSH3, PMS1, MSH3, CDKN2A (42), *FANCI*, *FANCA*, *FANCD2*, *RECQL4*, *RECQL5*, *BLM* (43).
RNA processing	*DIS3/RRP4*, *FAM46C* (33, 37, 39, 41–43), SF3B1 (39)
Cell cycle/mitosis/chromatin remodeling	*ATRX*, *NOP16*, *CECR5*, *MELK*, *TPX2*, *NCAPG2*, *CDK1*, *DTL* (51), *CDNK1B*, *FUBP1* (39, 41), ARID2, KDM6A, EP300, HIST1H1B, HIST1H1D, HIST1H1E, HIST1H2BK, *MMSET* (41), CDKN2C (41, 43) *CNOT3*, *KMT2D*, *MN1*, *EZH2* (42), *SETD2 PALB2*, *HDAC7*, *DOT1L* (42, 43), *TET2*, *PTPN11*, PRKD2 (39, 41, 43).
Oxidative stress	*ROMO1* (47), *BLVRB* (51).
Immune function	*KLHL6*, *IRF4*, *LTB*, *PRDM1* (39, 43).
Lipid metabolism	*AGPS* (51).

developed to test novel druggable targets. Such screenings have also the potential to establish putative novel regulators of immunomodulatory drug or proteasome inhibitor sensitivity.

Zhu et al. (53) transfected a library of 27968 RNAi in MM cells to determine lenalidomide sensitizers, identifying 63 genes that empowered lenalidomide activity upon silencing.

Among others, Ribosomal protein S6 kinase (RPS6KA3 or RSK2), five RAB family members, three potassium channel proteins, two peroxisome family members, I-k-B kinase-a (CHUK), and the transcription factor CREB1 were found the most sensitizing. Specific functional validation of RSK2 inhibition with RNAi or chemical inhibition not only sensitized MM cells to lenalidomide, but also to bortezomib, melphalan, or dexamethasone, pointing to a promising molecular target in MM therapy. Liu et al. (54) through genome wide CRISPR/CAS9 screening, identified seven out of nine of the CSN9 signalosome complex subunits as regulators of immunomodulatory drugs (IMIDs) sensitivity, by modulating the lenalidomide target Cerebron (CRBN) expression. Specific functional knock out of each of these *CSN* genes lead to partial pomalidomide and lenalidomide resistance, determining CRBN protein reduction.

Another example of how high-throughput research can help the identification of stress related targets, comes from the work of Stessman et al. (55) in which it was performed high-throughput drug screening in bortezomib sensitive and resistant cells. Among 1,600 small molecule compounds, 12 molecules were identified as effective in all the tested groups, among which four were toxic on bortezomib resistant cells or were able to restore their bortezomib sensitivity. The further functional assays were performed on the compound NSC622608, which was demonstrated to cause MM cell death through the modulation of TP53 signature, with the upregulation of the P53 dependent P21, NOXA, and PUMA proteins, the upregulation of *MT1H*, *HMOX1*, and *ANXA2* genes and the reduction of *POLD2*, *MCM5*, *MCM4*, *MCM3*, *MCM2*, *KIAA0101*, and *CCNA2* genes.

A tentative approach to link high-throughput drug screening with gene expression profiling and mutational analysis, has been presented at the 2019 ASH meeting. Coffey et al. (56) tested simultaneously 170 compounds and their target inhibitors along with NGS profiling to predict sensitivity to drugs. The registered clinical trial NCT03389347 (https://clinicaltrials.gov/ct2/show/NCT03389347) will analyze the feasibility of using high-throughput drug sensitivity and genomics data to evolve personalized treatments.

RNAi, CRISPR/CAS9, and small molecule drug screening are therefore an emerging field for the discovery of MM vulnerable targets, but to date the results on stress related pathways are scarce and more experimentation is underway.

CAPITALIZING ON THE INFORMATION FROM "-OMICS" TOWARD ACTIONABLE TARGETS

The body of data obtained from high-throughput transcriptomic and genomic analyses has allowed to better elucidate the major pathobiological MM alterations. However, for these research achievements to be clinically useful, it is important to identify molecules/pathways most suitable for therapeutic targeting. In this regard, the recurrently altered mechanisms involved in

cellular stress resistance and resilience against certain pro-death stimuli, which have been described in MM, could represent a groundwork to design new therapies.

Targeting MM-Associated Anomalies in Molecules Involved in the Management of Protein Synthesis, ER Stress, and Autophagy

Malignant plasma cells are addicted to both UPR and autophagy and the inhibition of the proteasome is now a well-established step in the therapy of MM (57, 58). It is now believed that targeting UPR and/or autophagy regulating proteins may further contribute to MM cell apoptosis (9, 59, 60).

IRE1α is a peculiar enzyme endowed with a kinase and endoribonuclease activity (61, 62). It has been demonstrated that the levels of XBP1 transcription factor, which regulates the IRE1α-dependent branch of UPR, are important to confer bortezomib resistance (63). Indeed, the IRE1α-XBP1 axis seems a suitable therapeutic target for this disease (64–66). The inhibition of IRE1α endoribonuclease domain and therefore of XBP1 splicing was also proposed as a promising strategy to reduce the MM cell capacity of coping with the proteotoxic stress and kill MM cells (65, 66). The small compound STF-083010 displayed antimyeloma activity *in vitro* and *in vivo* (65) and the molecule MKC-3946 was able to stop the bortezomib and HSP90 inhibitors-induced ER stress with consequent increased cell death due to decreased XBP1 splicing and increased GAD153 levels (66). The potential beneficial effects of interrupting the IRE1α/XBP1 axis in MM have also been described in other studies, in which this pathway was impaired by manipulating upstream molecules acting as regulators (48).

The PERK/GADD153/eIF2α branch of the UPR is believed to regulate survival or apoptosis depending on the magnitude of its activation. Earlier studies demonstrated that it was possible to enhance the GADD153/eIF2α-dependent pro-apoptotic arm of the UPR by stopping eIF2αdephosphorylation (67). Using *in vitro* and *in vivo* models, it was shown that this perturbation of UPR was associated to a progressive elimination of bortezomib-resistant/G0-G1 cell cycle-arrested MM cells (67). In another study, it was shown that the down-modulation of the PERK axis causes a non-apoptotic cell death triggered by autophagy (68). Other means of perturbing this pathway have targeted ER stress/UPR upstream regulative kinases, such as CK2, with the result of causing a strong activation of PERK-mediated phosphorylation of eIF2α and consequent irreversible pro-apoptotic UPR (48). Interestingly, it was also shown that blocking the PERK or ATF4-elicited UPR may cause tumor growth arrest and a reduction of neoangiogenesis after glucose deprivation (69).

Targeting the chaperone machinery has also been therapeutically relevant in MM. Preclinical studies (48, 70–72) or clinical trials have been conducted using HSP90 inhibitors such as 17-AAG, NVP-AUY922, KW-2478 alone, or in association with bortezomib or dexamethasone in MM (ClinicalTrials.gov Identifier: NCT00514371 and NCT00546780 for 17-AAG, ClinicalTrials.gov Identifier: NCT00708292 for AUY922 and ClinicalTrials.gov Identifier: NCT01063907 for KW-2478). Moreover, it has been shown that HSP70, a protein which OMICS data have demonstrated to be altered across the stages of MM progression (see above), is a chaperone of HSP90, that mediates drug resistance in MM sustaining plasma cell survival. The concomitant inhibition of both HSP70 (with VER-155008) and HSP90 (with NVP-AUY922) increases MM cell death abolishing HSP70 upregulation induced by HSP90 inhibition and affecting PI3K-dependent MM survival signaling (73). HSP70 inhibition induced plasma cell apoptosis accumulating proteotoxic stress (17), causing changes in polyubiquitination, in ER stress/UPR protein expression, and chaperone related autophagy markers (such as LAMP-2A) (74–77). Therefore, a lot of efforts have been made to develop inhibitors that could target both chaperones. To this aim, CCT251236 or KRIBB11, novel Heat Shock Factor 1 (HSF1) inhibitors, have shown cytotoxic effects in MM cells, via induction of UPR, with altered EIF2α phosphorylation, CHOP expression and a reduction in protein synthesis (78). Thus, chaperone targeting seems a promising approach for the treatment of MM.

Autophagy inhibitors are also currently employed in clinical trials in MM. Hydroxychloroquine (HCQ)/Chloroquine(CQ) have been tested to increase the effects of proteasome inhibitors (bortezomib) preclinically (79) or in clinical trials underway in association with cyclophosphamide and dexamethasone (ClinicalTrials.gov Identifier: NCT01438177). Histone Deacetylases (HDAC) are deacetylating proteins that catalyze the excision of acetyl groups on Lys in given proteins, not limited to histones, providing further levels of control in protein homeostasis. HDAC6, in particular, has been involved in the autophagic process, by promoting aggresome formation and autophagosome-lysosome fusion (80). It has therefore been proposed to use HDAC6 inhibitors to interfere with autophagy and hamper protein homeostasis in MM. HDAC6 inhibitors such as WT161 and tubacin have displayed anti-MM cytotoxicity, modulating ER stress/UPR signaling events, overcoming proteasome inhibitors resistance (81, 82). Different HDAC inhibitors, such as panobinostat, a pan HDAC inhibitor, have been tested in clinical trials or have been approved in relapsed/refractory MM in association with bortezomib (83). Other more recently described autophagy modulators are protein kinases. In particular, our and others' laboratory work has described the role of protein kinase CK1α and CK1δ in the autophagic process in MM (84). It has been shown that the CK1α and CK1δ members of the CK1 family of S/T kinases may control the autophagic flux downstream oncogenic RAS as well as its tonic rate, thus impacting on the survival capability of MM plasma cells (84–86).

Targeting MM-Associated Anomalies in Molecules Involved in the Management of DNA Damage-Induced Stress

Recent work has highlighted the importance of replicative stress management for myeloma cell survival. Cottini et al. (20) described a subset of aggressive myeloma displaying DNA damage due to chronic replicative and oxidative stresses, in

part caused by the high activity of c-MYC. Remarkably, the concomitant inhibition of the DNA damage induced repair kinase ATR along with the blockade of ROS-triggered stress managing enzyme SOD, exerted a synthetic lethality on this aggressive subtype of MM cells (20).

In the paper by Viziteu et al., it has been demonstrated a dependence of MM cells on RECQ1 helicase, a DNA unwinding enzyme essential for chromosomal integrity. This enzyme is overexpressed in MM cells and protects from melphalan and bortezomib-induced DNA damage and cytotoxicity. Interestingly, through a miRNA-203-dependent pathway RECQ1 expression is downregulated after treatment with DNA methyl transferase inhibitors (21), thus representing a potential target for combined treatments.

CONCLUSIONS

The information generated by the different high-throughput research on molecules and pathways affected in MM pathogenesis and evolution, have allowed to depict a very complex and heterogeneous scenario. Within this picture, it has been possible to identify some common alterations in cellular molecules/processes/mechanisms, which belong to the stress-related homeostatic response. It is becoming increasingly clear that some of these processes may be efficiently targeted for a therapeutic purpose, especially in combination with other approaches (**Figure 1**). Future research should focus on these molecules and validate their targeting as effective to achieve a clinically meaningful anti-MM action.

AUTHOR CONTRIBUTIONS

FP and SM conceived and wrote the paper, collected data, and envisaged the format. AF, GB, RZ, and GS wrote parts of the paper, contributed data, and insights.

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Down-Regulated FOXO1 in Refractory/Relapse Childhood B-Cell Acute Lymphoblastic Leukemia

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Background: Acute lymphoblastic leukemia (ALL) is the most common pediatric cancer, with an overall prevalence of 4/100,000, accounting for 25–30% of all childhood cancers. With advances in childhood ALL treatment, the cure rate for childhood ALL has exceeded 80% in most countries. However, refractory/relapsed ALL remains a leading cause of treatment failure and subsequent death. Forkhead box O1 (FOXO1) belongs to the forkhead family of transcription factors, but its role in B-cell ALL (B-ALL) has not been determined yet.

Procedures: RNA sequencing was applied to an ALL case with induction failure (IF) to identify the possible genetic events. A cytokine-dependent growth assay in Ba/F3 cells was used to test the leukemic transformation capacity of MEIS1–FOXO1. The propidium iodide (PI) staining method was used to evaluate the effect of MEIS1–FOXO1 on cycle distribution. FOXO1 transactivity was examined using a luciferase reporter assay. *FOXO1* mRNA expression levels were examined using real-time quantitative PCR among 40 children with B-ALL treated with the CCCG-ALL-2015 protocol. Association analysis was performed to test the correlation of *FOXO1* transcription with childhood B-ALL prognosis and relapse in a series of GEO datasets. An MTT assay was performed to test the drug sensitivity.

Results: In this ALL case with IF, we identified a novel *MEIS1–FOXO1* fusion gene. The transactivity of MEIS1–FOXO1 was significantly lower than that of wild-type FOXO1. *MEIS1–FOXO1* potentiated leukemia transformation and promoted Ba/F3 cell cycle S-phase entry. Low *FOXO1* transcription levels were found to be strongly associated with unfavorable ALL subtype, minimal residual disease (MRD) positivity, and relapse. Lower *FOXO1* expression was associated with prednisone and cyclophosphamide resistance.

Conclusions: Low *FOXO1* transcription was associated with high-risk stratification and relapse in children with B-ALL, probably due to multi-drug resistance.

Keywords: acute lymphoblast leukemia, risk stratification, relapse, *FOXO1*, MRD—minimal residual disease

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common pediatric cancer, with an overall prevalence of 4/100,000, accounting for 25–30% of all childhood cancers (1). The cure rate for childhood ALL has exceeded 80% in most countries and even higher than 90% in developed countries with contemporary therapy (2). However, refractory/relapsed ALL (R/R-ALL) remains to be a leading cause of treatment failure and subsequent death (3). Although most patients can achieve quick and long-term responses to contemporary chemotherapy, a non-ignorable portion of childhood ALL patients do not respond well or relapse during chemotherapy. Cumulating evidence has pointed out that the long-term outcome of patients with relapsed or induction failure (IF) is very dismal. Thus, precise risk stratification at an early stage is very essential for directing patients into more optimized therapy regimens.

Importantly, genomic lesions play a deterministic role in R/R-ALL. For example, patients with Philadelphia chromosome (Ph) translocation, *PDGFRB*-rearrangement, *MEF2D*-rearrangement, *KMT2A*-rearrangement, *TP53* mutation, and *TCF3-HLF* are classified into high- or very high-risk ALL subgroups (4). Meanwhile, the gene expression profile (e.g., Ph-like signature) can predict the therapeutic response and relapse of ALL (5). Many findings have been translated into drug discovery, which improved clinical application. An example of the result of such finding is the milestone BCR-ABL1 targeted tyrosine kinase inhibitor, imatinib (6). However, a considerable portion of clinical failure cannot be entirely explained by our current knowledge. Thus, studies on ALL biology and refractoriness/relapse prediction are necessary for the early identification of new driver alterations and subsequent treatment with more aggressive strategies, such as chimeric antigen receptor (CAR) T-cell therapy or hematopoietic stem cell transplantation (HSCT) (7).

In this study, we identified a novel forkhead box O1 (*FOXO1*) fusion gene, namely, *MEIS1–FOXO1*, in a B-cell ALL (B-ALL) case with IF. Using the Ba/F3 transformation model, we found that *MEIS1–FOXO1* could potentiate leukemogenesis *in vitro* and cell cycle S-phase entry. Furthermore, the transcription activity of the MEIS1–FOXO1 fusion protein was completely abolished as compared with its wild-type FOXO1 protein. Gene expression correlation analysis identified that lower *FOXO1* transcription levels were associated with high-risk stratification and relapse in children with B-ALL. Finally, we tested the role of FOXO1 in drug response and found that lower *FOXO1* expression was associated with prednisone and cyclophosphamide resistance.

PATIENTS AND METHODS

Patients

The patients were prospectively enrolled in the CCCG-2015-ALL clinical trial, which was approved by the institutional review board of the Guangzhou Women and Children Medical Center (GWCMC) (2018022205). Details of the enrollment criteria and study design have been described previously (8). All the investigated pediatric ALL patients were treated in the GWCMC. This study was approved by the Institutional Ethics Committee of the GWCMC (IRB nos. 2018022205, 2017102307, 2015020936, and 2019-04700), registered at the Chinese Clinical Trial Registry (ChiCTR-IPR-14005706), and conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from the patients or their legal guardians.

Next Generation Sequencing and Validation

TruSeq stranded mRNA library prep kit (Illumina) was used for whole-transcriptome library preparation, and paired-end sequencing was performed using the Illumina HiSeq 2,000/2,500 platform with a 101-bp read length at Berry Genomics, Beijing. Panel sequencing of hematological malignancy-related genes (**Supplementary Table 1**) was performed at Kindstar Global (Beijing) Technology, Inc. Sequencing reads were aligned to the human genome (hg19) reference sequence using TopHat2 (v2.0.12) (9). *MEIS1–FOXO1* fusion was validated by PCR amplification of breakpoint region of the chimeric transcript in this patient's cDNA using primers listed in **Supplementary Table 2**, followed by Sanger sequencing.

Cytokine-Dependent Growth Assay in Ba/F3 Cells

Full-length *FOXO1*, *MEIS1*, and *MEIS1–FOXO1* were amplified and cloned into the cL20c-IRES-GFP lentiviral vector. Lentiviral supernatants were produced by transient transfection of HEK-293T cells using calcium phosphate. The MSCV-JAK2^{R683G}-IRES-GFP construct was a gift from Dr. Jun Yang at St. Jude Children's Research Hospital (10). It was modified into MSCV-JAK2^{R683G}-IRES-mCherry, and retroviral particles were produced using 293T cells. Ba/F3 cells were maintained in a medium supplemented with 10 ng/ml recombinant mouse interleukin 3 (IL-3) (PeproTech). Ba/F3 cells were transduced with lentiviral supernatants expressing *FOXO1, MEIS1*, or *MEIS1–FOXO1*. GFP-positive cells were sorted 48 h after lentiviral transduction and maintained in an IL-3 medium for another 24 h before transfection with *JAK2^{R683G}* retroviral supernatants. Forty-eight hours later, GFP/mCherry double-positive cells were sorted and maintained in a medium with respective cytokines for 48 h. Then, the cells were washed three times and grown in the absence of cytokines. Cell viability was monitored daily with Trypan blue using a TC10 automated cell counter (BIO-RAD). Each experiment was performed in triplicates.

Luciferase Reporter Assays

The full-length *FOXO1*, *MEIS1*, and *MEIS1–FOXO1* were amplified and cloned into the cL20c-IRES-GFP lentiviral vector and used for luciferase reporter assays to test their transactivation capability on the genes with conserved FOXO1 binding sites in HEK-293T cells. Lentiviral vectors expressing *MEIS1–FOXO1* and pGL.3 reporter constructs containing FOXO1 binding sites were co-transfected into HEK-293T cells. Cells were lysed 24 h after transfection with passive lysis buffer (Promega, E1910). Luciferase activity was measured using a dual-luciferase reporter assay on a Lumat LB9507 luminometer. Experiments were

performed in triplicates. To control for cell number and transfection efficiency, firefly luciferase activity was normalized to Renilla luciferase.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted using the RNeasy Micro kit (Qiagen) according to the manufacturer's protocol. Five hundred nanograms of total RNA from patient samples (**Supplementary Tables 3, 4**) was reverse transcribed into cDNA, and real-time quantitative PCR (qRT-PCR) was performed using an ABI Prism 7900HT detection system (Applied Biosystems) with FastStart SYBR Green Master mix (Roche). *GAPDH* was used as an internal control. Primers used were listed in **Supplementary Table 2**.

Gene Expression Analysis

The raw gene expression data and clinical data from four cohorts of childhood ALL patients were provided by other research groups (11–15). The relative gene expression levels (fragments per kilobase of transcript per million mapped reads, FPKM) were estimated based on supporting reads retrieved from the datasets. The FPKM values were $\log_2$ transformed for subsequent analyses and plotting. A two-sided *t*-test was used to validate the significance of the observed differences.

In vitro Cytotoxicity Assay

Cells were seeded in 96-well plates at 25,000 cells per 100 μl per well with either vehicle (DMSO 0.1%) or increasing concentrations of drugs for 72 h. Cell viability was assessed by adding MTT reagent (Sigma) according to the manufacturer's instructions. Procedures to determine the effects of certain conditions on cell proliferation were performed in three independent experiments.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism® and/or R (version 3.2.5, https://www.R-project.org); all tests were two-sided. $P < 0.05$ was considered to be statistically significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

RESULTS

Identification of a Novel *MEIS1–FOXO1* Fusion Gene in a B-ALL Case With Induction Failure

A total of 466 children with ALL were enrolled from March 2015 to June 2020 in the CCCG-ALL-2015 study of Guangzhou Women and Children's Medical Center, of which 427 were children with B-ALL. Around 0.94% (4 out of 427) of enrolled B-ALL patients did not respond to induction remission therapy and were classified as IF ALL (median age, 8.6 years; range, 2.1–11.9 years) (**Figure 1**), which was consistent with reports from other study groups (16). Among these, three IF cases were found to have known fusion genes (i.e., *MEF2D-BCL9*, *TCF3-HLF*, and *ZC3HAV1-ABL2,* respectively). The *MEF2D-BCL9* and *TCF3-HLF* fusion genes are well-established and classified into poor prognostic ALL subtypes (17–19), and *ZC3HAV1-ABL2* was designated as Ph-like subtype (5). Interestingly, this 2.1-year-old precursor B-ALL boy with IF could not be explained by known molecular events contributing to this treatment response (**Supplementary Figure 1**). At the end of induction remission therapy, 20.5% of lymphoblastic cells were detected in the bone marrow smear samples, and the minimal residual disease (MRD) level detected by flow cytometry was 10.3% (**Supplementary Figure 1A**). Regular pathology tests showed that he was a B-cell precursor ALL (BCP-ALL) patient with abnormal 46,XY,del(17)(p11)[15]/46,XY,i(17)(q10)[2]/46,XY[3] karyotype. Using a capture sequencing, we also identified the $NRAS^{G12D}$, $TP53^{R273H}$, $ABCC1^{R1176X}$, $PHGR1^{H37P}$, $HOXA3^{P219L}$, and DST^{P4606L} mutations. No fusion gene was identified using the current panel RT-PCR assay (**Supplementary Figures 1C–E**). To determine the possible cause of IF, we performed RNA sequencing and found a novel *MEIS1–FOXO1* fusion gene that was an in-frame fusion of exon 1–6 of *MEIS1* with exon 2 of the *FOXO1* gene, which was confirmed by RT-PCR and Sanger sequencing (**Figures 2A,B** and **Supplementary Figures 2A,B**).

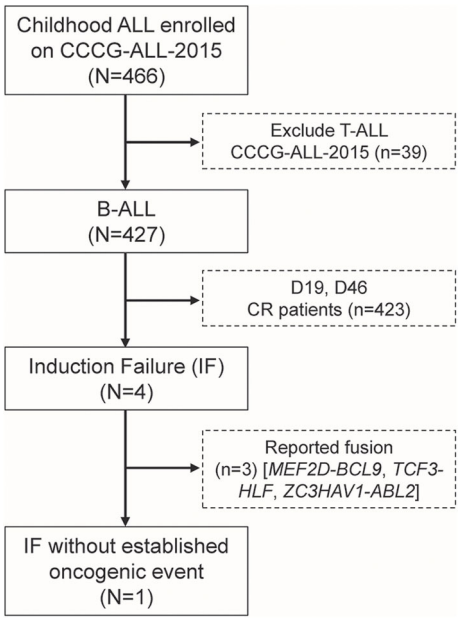

FIGURE 1 | Identification of one induction failure B-ALL case without known fusion gene among patients enrolled onto CCCG-ALL-2015 in Guangzhou Women and Children's Medical Center.

The Oncogenic Potential of *MEIS1–FOXO1*

These findings prompted us to ask whether this novel *MEIS1–FOXO1* fusion gene drives B-ALL leukemogenesis and contributes to poor treatment response. To address this question, we used an IL-3-dependent growth mouse hematopoietic progenitor cell line Ba/F3 as a study model and tested whether *MEIS1–FOXO1* had some kind of oncogenic potential. Since somatic *NRAS* mutations have been reported to be

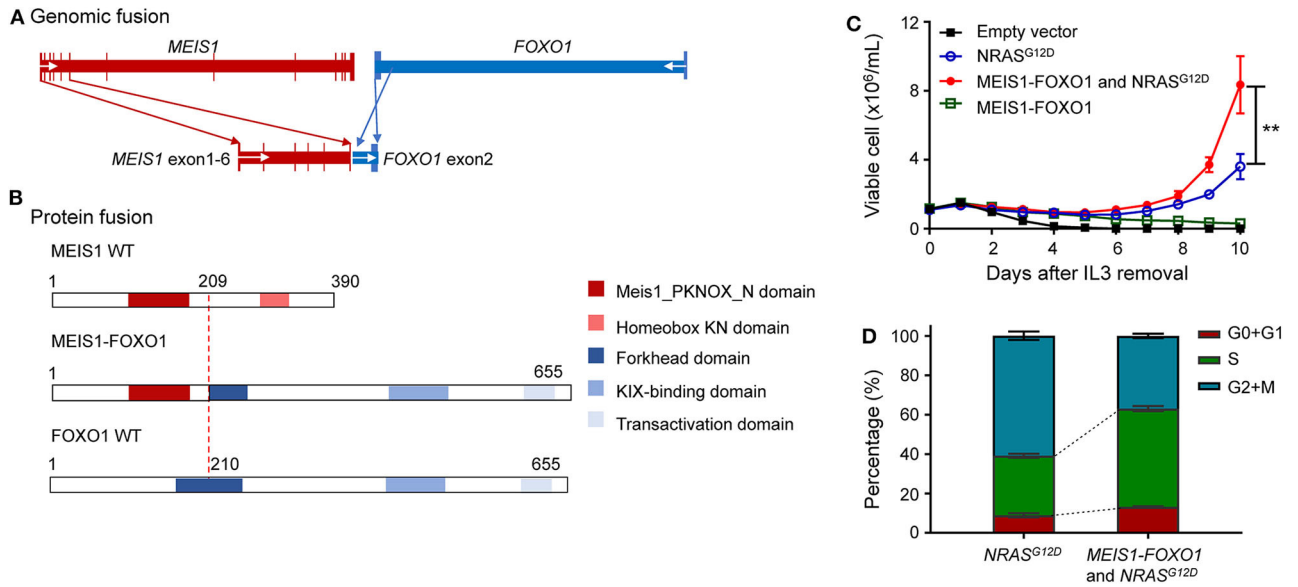

FIGURE 2 | Identification of oncogenic potential of *MEIS1–FOXO1* fusion gene. **(A)** Schematic DNA representation of novel *MEIS1–FOXO1* rearrangement (red, *MEIS1*; blue, *FOXO1*). **(B)** Fusion protein representation of novel *MEIS1–FOXO1* rearrangement (red box, MEIS1 PKNOX N domain; pink box, homeobox KN domain; dark blue box, forkhead domain; light blue box, KIX-binding domain; gray blue box, transactivation domain). **(C)** MEIS1–FOXO1 potentiated leukemia transformation in Ba/F3 cell model. Effects of *MEIS1–FOXO1* fusion genes on Ba/F3 transformation. Following transduction of empty vector, *MEIS1–FOXO1*, $NRAS^{G12D}$, or combination, Ba/F3 cells were cultured in IL-3 depleted medium with cytokine-independent cell growth as a measure of oncogenic transformation. Number of viable cells was evaluated daily. **(D)** MEIS1–FOXO1 fusion genes and proliferation of mouse hematopoietic progenitor cell Ba/F3. Ba/F3 cells were lentivirally transduced with empty vector (black), $NRAS^{G12D}$ (blue), *MEIS1–FOXO1* (green), or combination of $NRAS^{G12D}$ and *MEIS1–FOXO1* (red) and then cultured in the presence of IL-3 (10 ng/ml). After 48 h, cell cycle distribution was evaluated using standard PI staining protocol. Statistical significance, determined using the two-sided unpaired *t*-test, is indicated by $^{**}P < 0.01$.

sufficient for transforming leukemogenesis, we used $NRAS^{G12D}$ as our experimental control. Consistent with the reports by Shannon and Castilla (20, 21), our *in vitro* assay showed that ectopic $NRAS^{G12D}$ expression potentiated Ba/F3 cells IL-3-independent growth (**Figure 2C**). Although MEIS1–FOXO1 was not sufficient to transform Ba/F3 cells into IL-3-independent growth, it indeed potentiated the survival of Ba/F3 cells compared with cells transfected with mock vector (**Figure 2C** and **Supplementary Figure 3**). Furthermore, the combination of MEIS1–FOXO1 and $NRAS^{G12D}$ accelerated Ba/F3 cells into IL-3-independent growth as compared with $NRAS^{G12D}$ alone (**Figure 2C**). Using the same cell model, we tested the impact of MEIS1–FOXO1 on cell cycle distribution and found that the cotransduction of MEIS1–FOXO1 and $NRAS^{G12D}$ potentiated S-phase entry in comparison with $NRAS^{G12D}$ alone (**Figure 2D**). These results suggest the oncogenic potential of *MEIS1–FOXO1*.

Lower *FOXO1* Transcription in This Induction Failure B-ALL Case

Accumulating evidence has demonstrated that *FOXO1* is a crucial regulator of B-cell development, in which FOXO1 inactivation causes differentiation blockage at the pro-B-cell stage (22–25). To investigate the role of *MEIS1–FOXO1* in B-ALL, we first examined the gene expression of fusion partners in normal hematopoiesis and B-ALL patient samples. As shown in **Figure 3A**, a gradual up-regulation of *FOXO1* expression was observed during B-cell differentiation, whereas *MEIS1* was downregulated, suggesting an important role of *FOXO1* in B-cell development (**Figure 3A** and **Supplementary Figure 4A**). In B-ALL samples, we found that *FOXO1* was constitutively expressed in B-ALL cells, whereas *MEIS1* was merely expressed, again suggesting the role of *FOXO1* in B-ALL (**Figure 3B** and **Supplementary Figures 4B,C**). To test the biological functions of *MEIS1–FOXO1*, we first utilized a luciferase reporter assay to determine the impact of MEIS1–FOXO1 on the transactivity. As shown in **Figure 3C** and **Supplementary Figure 5**, FOXO1 transactivity was abolished entirely in MEIS1–FOXO1 as compared with wild-type FOXO1. We then tested whether MEIS1–FOXO1 had a dominant-negative effect on its wild-type FOXO1 protein. As shown in **Figure 3C**, no dominant-negative effect was observed. Furthermore, we quantified the *FOXO1* gene expression using RT-qPCR and found that *FOXO1* was nearly not expressed in the leukemic cells of this patient with MEIS1–FOXO1 fusion (**Figure 3D**). Together, low *FOXO1* transcription might have contributed to IF in this patient.

Lower *FOXO1* Transcription Might Be Associated With Poor Outcomes in Children With B-ALL *via* Drug Resistance

These findings prompted us to ask whether *FOXO1* expression was associated with the prognosis of B-ALL. To test our hypothesis, we retrieved and analyzed the *FOXO1* expression data from the Pediatric Cancer Genome Project (PCGP) (26) and found that *FOXO1* gene expression was the highest

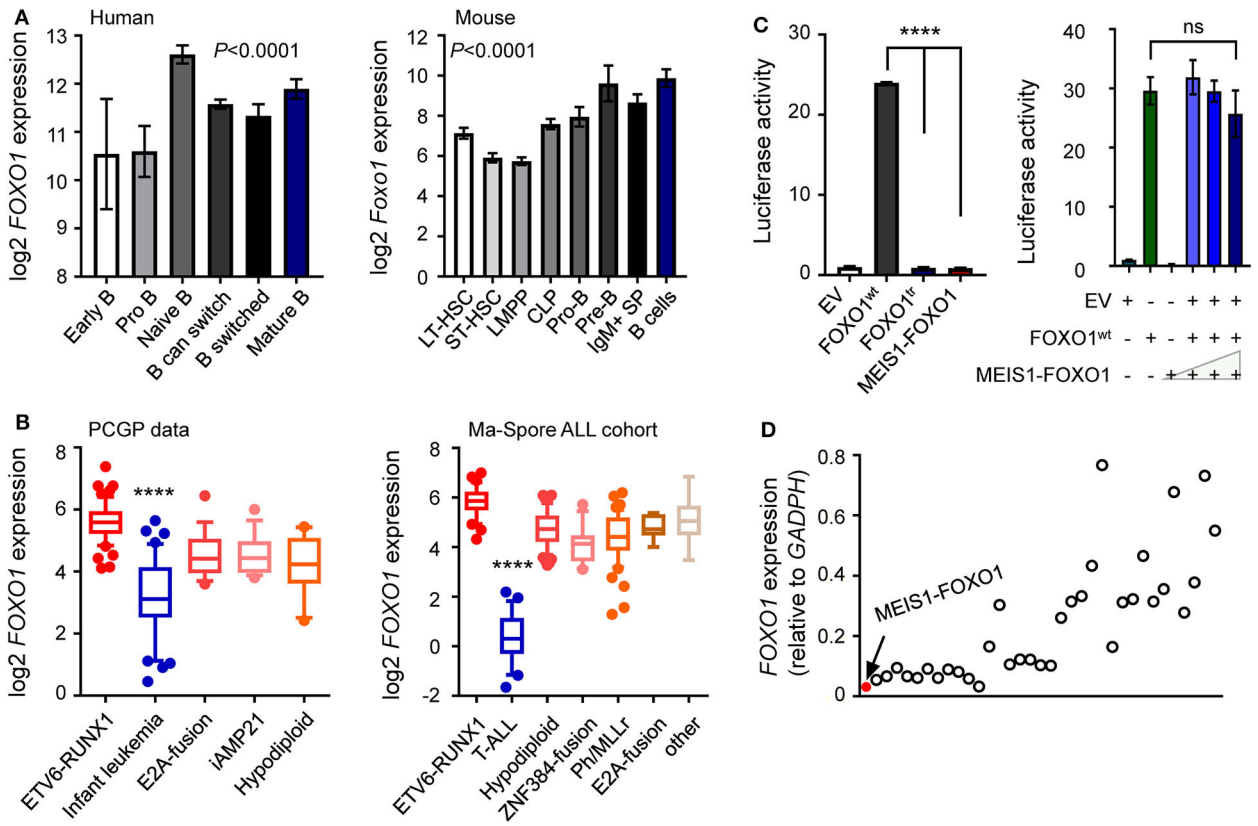

FIGURE 3 | Lower *FOXO1* transcription associated with poor prognosis. **(A)** Expression of the *FOXO1* was increased along with human (left panel) and mouse (right panel) B lymphocyte development. Each B-cell group is represented by a bar and is color-coded according to the subgroups it belongs to. **(B)** Expression of the *FOXO1* was constitutively activated among B-ALL samples, with the highest expression in *ETV6-RUNX1* subtype. **(C)** Luciferase reporter gene assay of MEIS1–FOXO1 transcription activity. HEK-293T cells were transiently transfected with pGL3 construct (luciferase gene with FOXO1 binding sites), pcDNA construct [empty vector, wild-type FOXO1 [FOXO1^{wt}], MEIS1–FOXO1, or truncated FOXO1 [FOXO1^{tr}]], and pGL-TK (Renilla luciferase). **(D)** Expression of the *FOXO1* among 35 B-ALL cases. RT-PCR was performed to quantify the *FOXO1* transcription, and the quantification was expressed as relative to internal *GAPDH* control. The red dot represented the B-ALL case with *MEIS1–FOXO1* fusion gene. Statistical significance, determined using one-way ANOVA test **(A,B)** or two-sided unpaired *t*-test **(C)**, is indicated by ****$P < 0.0001$.

in *ETV6-RUNX1* ALL and lowest in infantile leukemia. Notably, *FOXO1* expression was significantly lower in patients with intermediate or high-risk ALL than in *ETV6-RUNX1* ALL, a well-known excellent prognosis group (**Figure 3B** and **Supplementary Figure 6**). Next, we applied the same strategy to analyze the data from the Ma-Spore ALL cohort (27) and observed the same pattern (**Figure 3B**). Moreover, in the Ma-Spore ALL cohort, we found that lower *FOXO1* expression was associated with higher MRD burden post-induction remission therapy ($P = 3.8 \times 10^{-4}$ by Wilcoxon rank-sum test, **Figure 4A**).

To evaluate the impact of *FOXO1* on B-ALL relapse, we examined the *FOXO1* mRNA levels in diagnostic and relapsed B-ALL samples from our single institution using RT-qPCR assay. As shown in **Supplementary Figure 7A**, *FOXO1* transcription was significantly higher in the diagnosed ALL samples than in the relapsed samples, suggesting that lower *FOXO1* transcription might be an essential index for B-ALL relapse. This expression pattern was also observed in the St. Jude PCGP dataset (**Supplementary Figure 7B**). To further validate this finding, we next tested the *FOXO1* transcription among five paired diagnosis-relapse samples in our study cohort and identified an extremely low *FOXO1* expression in the relapsed samples as compared with their diagnostic counterparts (**Figure 4B**). This observation was validated in the PCGP and the matched diagnosis-relapse dataset created by Hogan et al. (13) (**Figures 4C,D**), consolidating the role of *FOXO1* in B-ALL relapse. To preliminarily explore how lower *FOXO1* expression is linked with higher MRD levels and relapse, we performed a drug resistance association analysis on the datasets published by Paugh et al. (28). Of note, lower *FOXO1* expression significantly correlated with glucocorticoid resistance (**Figure 4E**), a key component in ALL therapy. Next, we knocked down *FOXO1* expression in Nalm6, a B-ALL leukemia cell line, and then examined the drug response. As shown in **Figure 4F**, Nalm6 cells with lower *FOXO1* transcription were relatively resistant to prednisone (IC50 = 1.1 μM in *FOXO1* knockdown and 0.18 μM in Nalm6 cells) and cyclophosphamide (IC50 = 4.6 μM in *FOXO1* knockdown and 1.0 μM in Nalm6 cells). Using the Ba/F3 cell model, we tested the role of *MEIS1–FOXO1* in drug response. Consistent with the role

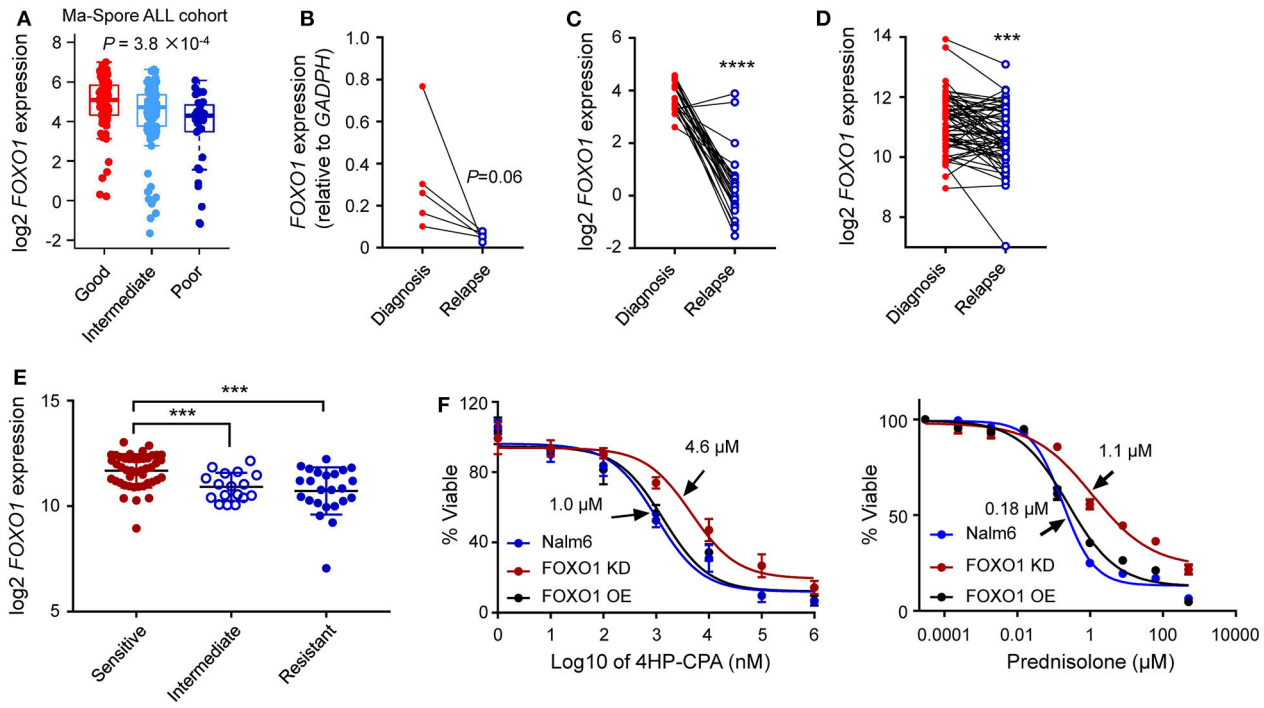

FIGURE 4 | Lower *FOXO1* transcription correlated with ALL relapse. **(A)** Lower *FOXO1* was associated with MRD positivity at the end of induction. MRD at the end of induction therapy Day 33 was used: patients with MRD <0.01% or >1% were classified as "good" or "poor," respectively, and others were classified as "intermediate." **(B,C)** Lower *FOXO1* expression was found in relapsed B-ALL as compared with primary samples in our institutional data **(B)** and PCGP data **(C)**. **(D)** The FOXO1 transcription was extremely low in relapsed B-ALL among the diagnosis-relapse matched paired samples. **(E)** Lower FOXO1 expression was associated with glucocorticosteroids resistance. **(F)** Cytotoxicity of prednisone and 4HP-CPA was examined in Nalm6 cells with FOXO1 knockdown (dark red line, FOXO1 KD), over-expression (black line, FOXO1 OE), or parental cells (blue line). Cells were incubated with drugs for 72 h, and viability was then measured using MTT assay. Experiments were performed in triplicate and repeated at least three times. Statistical significance, determined using two-sided unpaired *t*-test **(A,E)** or two-sided paired *t*-test **(B–D)**, is indicated by ***$P < 0.001$ and ****$P < 0.0001$.

of FOXO1, the introduction of *MEIS1–FOXO1* also induced cyclophosphamide resistance in Ba/F3 cells transformed by $NRAS^{G12D}$. However, the impact on prednisone resistance was very moderate (**Supplementary Figure 8**).

DISCUSSION

R/R-ALL is the priority issue for clinicians and translational researchers. Multiple layers of influencing factors, that is, specific somatic genomic lesions, inherited variation, micro-environment, and acquired mutations, play essential roles in R/R-ALL. In this study, we identified a novel *FOXO1* fusion gene in an IF B-ALL patient, namely, *MEIS1–FOXO1*. The frequency of MEIS1–FOXO1 in our study cohort is 0.23%. In the PCGP dataset, we have found one case (0.17%) with MEIS1–FOXO1 fusion out of 565 B-ALL cases. Except for the novel fusion gene, we have identified several gene mutations (i.e., $ABCC1^{R1166X}$, $HOXA3^{P219L}$, DST^{P4606L}, $NRAS^{G12D}$, and $TP53^{R273H}$) (**Supplementary Figures 1, 2**), among which, the role of $ABCC1^{R1166X}$, $HOXA3^{P219L}$, and DST^{P4606L} is less known in the context of childhood ALL. Regarding $NRAS^{G12D}$ and $TP53^{R273H}$ mutations, Irving et al. (29) have reported that *NRAS* and *TP53* mutations were associated with an increased risk of progression.

As a novel fusion gene, *MEIS1–FOXO1* has oncogenic potential, as evidenced by the fact that it prolonged Ba/F3 survival independent of IL-3 when transduced alone, accelerated Ba/F3 cell leukemic transformation, and potentiated cell S-phase entry when co-transduced with $NRAS^{G12D}$ as compared with transduction of $NRAS^{G12D}$ alone. We also noticed that the MEIS1–FOXO1 protein did not negatively impair wild-type FOXO1 protein function, and *FOXO1* was nearly not expressed in this patient, indicating that low FOXO1 expression might be the cause of IF. However, the exact molecular impact of MEIS1–FOXO1 in B-ALL leukemogenesis and development needs to be explored in the future.

Recent studies have shown that FOXO1 is a predominant transcription factor in B-lineage-restricted progenitor cells. Pre-BCR signaling activation can suppress FOXO1 transcription activity and subsequent B-ALL cell maintenance (30). The novel MEIS1–FOXO1 fusion protein was deficient in binding FOXO1-regulated genes but did not affect wild-type FOXO1 protein in a dominant-negative fashion *in vitro*, suggesting that the wild-type allele can be functional. Interestingly, we noticed that the FOXO1 expression was deficient in this patient with MEIS1–FOXO1 fusion, suggesting an underlying mechanism *in vivo* mediating the low expression of the wild-type allele of *FOXO1*. Notably, we also found that there was a correlation between

low FOXO1 transcription and ALL relapse among the enrolled patients and subjects in public datasets, and FOXO1 transcription was almost silenced in those relapsed patients as compared with their diagnostic counterparts, suggesting that FOXO1 status can be a valuable prognostic feature in ALL. Of note, our results showed that the FOXO1 transactivity was almost completely abolished in the MEIS1–FOXO1 protein, whereas its MEIS1 transactivity seemed regular, suggesting that MEIS1 may not play an essential role in ALL pathogenesis in this context.

FOXO1 belongs to the forkhead family of transcription factors, which play roles in myogenic growth and differentiation, cancer development, and therapy (31–34). Fusions of *FOXO1* have been found in pediatric alveolar rhabdomyosarcoma and childhood B-ALL (35). Interestingly, while two BCP-ALL cases with *FOXO1* fusion have been reported, the exact fusion partner and the role of *FOXO1* in B-ALL remain unclarified (36). To address this question, we identified a novel *MEIS1–FOXO1* fusion by RNA-seq and examined the association of FOXO1 expression with risk stratification in multiple datasets. We found that the FOXO1 was highly expressed in ALL patients with ETV6-RUNX1 fusion (a well-known excellent prognosis group) and significantly low expression in ALL subtypes with known poor prognosis (e.g., infantile leukemia, KMT2A-rearranged, and T-ALL), and this pattern was confirmed in different cohorts (33). We also observed a correlation between lower FOXO1 expression and higher MRD burden post-induction therapy ($P = 3.8 \times 10^{-4}$). Intriguingly, we found that lower FOXO1 expression was significantly associated with glucocorticoid resistance, a crucial component in the ALL therapy, which may explain how lower FOXO1 expression contributes to higher MRD levels and relapse.

In conclusion, we identified a novel fusion gene of *MEIS1–FOXO1* and first reported the association of reduced *FOXO1* expression with ALL high-risk stratification and relapse. Our findings suggest that FOXO1 status may be a predictive marker for B-ALL risk stratification and relapse.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found at: https://www.ncbi.nlm.nih.gov/geo.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Ethics Committee of Guangzhou Women and Children's Medical Center. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

HZ, CJ, HL, MQ, and QZ designed the research, analyzed the results, and wrote the paper. HZ, CJ, ZL, and JQ performed the experiments. HZ, PW, WH, HH, and QZ recruited and followed up the patient. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.579673/full#supplementary-material

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REVIEW
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DNA Copy Number Changes in Diffuse Large B Cell Lymphomas

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Copy number aberrations (CNV/CNA) represent a major contribution to the somatic mutation landscapes in cancers, and their identification can lead to the discovery of oncogenetic targets as well as improved disease (sub-) classification. Diffuse large B cell lymphoma (DLBCL) is the most common lymphoma in Western Countries and up to 40% of the affected individuals still succumb to the disease. DLBCL is an heterogenous group of disorders, and we call DLBCL today is not necessarily the same disease of a few years ago. This review focuses on types and frequencies of regional DNA CNVs in DLBCL, not otherwise specified, and in two particular conditions, the transformation from indolent lymphomas and the DLBCL in individuals with immunodeficiency.

Keywords: copy number aberrations, genetic alteration, lymphoma, diffuse large B cell lymphoma, hematological malignancies, MYC, TP53, CDKN2A

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INTRODUCTION

Copy number aberrations (CNV/CNA) represent a major contribution to the somatic mutation landscapes in cancers, and their identification can lead to the discovery of oncogenetic targets as well as improved disease (sub-) classification (1, 2). In malignant lymphomas, the contribution of partial and complete chromosomal CNV had been recognized early on through cytogenetic analyses (3, 4) and interphase fluorescence in-situ hybridization (FISH) studies (5, 6). The more systematic, genome-wide mapping of CNVs has been facilitated through the development of chromosomal comparative genomic hybridization (CGH) (7, 8) followed by array-based CGH technologies (aCGH) (9, 10) with increasingly higher spatial resolution, as well as through the widespread adoption of SNP-arrays (11) for copy number profiling. More recently the application of high throughput sequencing approaches (12, 13) has led to increasingly precise identification of regional gains or losses of genomic material (14–21), although the frequently used whole-exome sequencing strategies (WES) have limited precision for CNV mapping (22) compared to high-resolution genomic array technologies or whole-genome sequencing WGS). Diffuse large B cell lymphoma (DLBCL) is the most common lymphoma in Western Countries and up to 40% of the affected individuals still succumb to the disease (23–26). DLBCL is an heterogenous group of disorders as it has been demonstrated by studies that have explored transcriptome profiles and/or at DNA alterations in large series of cases (2, 3, 5, 7, 12, 17, 19, 23, 27–40). It is important to mention that the disease we call DLBCL is not necessarily the same of what we called DLBCL just a few years ago. Indeed, the so called "double" or "triple hit lymphomas", a subgroup of cases with particularly poor prognosis and previously largely included within DLBCL, are now regarded a distinct entity ("High-

grade B-cell lymphoma with MYC and BCL2 and/or BCL6 rearrangements") separate from the "DLBCL, not otherwise specified (NOS)" as expressed in the 2017 WHO classification (24, 31, 39, 41–43). A similar path was previously followed for primary mediastinal B-cell lymphoma (PMBCL), which, based on its very peculiar features (44, 45), was separated from DLBCL and it is considered a distinct clinicopathologic entity (24). Here, we will review the DLBCL genomics with a particular focus on types and frequencies of regional DNA CNVs in DLBCL, not otherwise specified and in two particular conditions, the transformation from indolent lymphomas and the DLBCL in individuals with immunodeficiency.

CNVS AND DLBCL

Within DLBCL, at least two main subtypes have been recognized, in which the gene expression profiles show similarities with two types of normal B-cells: the germinal center B-cell like (GCB) subtype and an activated B cell-like (ABC) subtype (15, 46–51). Clinically, those subtypes are characterized by prognostic differences; patients with an ABC DLBCL have a worse outcome than those with GCB DLBCL when treated with the standard chemo-immunotherapy chemotherapy regimen R-CHOP (24, 48, 49). Genetically, GCB and ABC DLBCL present a series of subtype-specific lesions that explain can explain the different biology of the disease, but they also share others that, with a couple of exceptions (*BCL6* and *MEF2B* alterations), are not DLBCL specific and can be observed in other lymphoma types or even in other cancers. Both GCB and ABC DLBCL present genetic alterations on genes encoding chromatin modifiers [*KMT2D*/MLL2 or *KMT2C*/MLL3 (mutations); *CREEBBP* (mutations or 16p13 deletions) or *EP300* (mutations or 22q13 deletions)], the germinal center master regulator BCL6 (*BCL6* chromosomal translocations, *MEF2B* mutations), proteins involved in DNA damage response ([*TP53* (mutations or 17p13 deletions)], or proteins contributing to immune surveillance [*B2M* (mutations or 15q21 deletions); *CD58* (mutations or 15q21 deletions)]. ABC DLBCL is characterized by lesions in genes involved in NF-κB pathway and B-cell receptor (BCR) signaling [*TNFAIP3* (mutations or 6q23 deletions); *MYD88*, *CD79A*, *CD79B*, *CARD11* (mutations)], cell cycle [*CDKN2A/B* (9p21 deletions)], terminal B cell differentiation [*PRDM1* (mutations or 6q21 deletions); *SPIB* (19q13 gains and amplifications)], and apoptosis [*BCL2* (18q21 gains or amplifications)]. In addition, ABC DLBCL have common gains affecting chromosome 3, which could might contribute to immune escape (*FOXP1*, 3p14), NF-κB pathway activation (*NFKBIZ*, 3q12) and B cell differentiation arrest (*BCL6*, 3q27) (4, 7, 13, 15, 17, 27, 30–32, 34, 36, 38, 40, 48, 49, 52–54). GCB DLBCL presents lesions leading to deregulated cell motility [*GNA13* (mutations)], apoptosis [*BCL2* (chromosomal translocations)], cell cycle [*MYC* (chromosomal translocations)], chromatin regulation [*EZH2* (mutations)], immune escape *TNFRSF14* (mutations or 1p36 deletions), PI3K/AKT signaling [*PTEN* (10q23 deletions); *MIR17HG* (13q31 gains or amplifications)], and DNA damage response [*ING1* (deletions)]. As for ABC DLBCL, also GCB DLBCL present some recurrent gains affecting specific (gains of 2p16 with *REL*) or large and still not fully characterized regions (chromosomes 7 and 12) (15, 16, 49–51, 55, 56). **Figure 1** shows examples of genomic profiles obtained in DLBCL.

The inferior outcome given by the ABC COO alongside the discovery of pathways specifically deregulated in this subtype led to clinical studies designed to target the activation of NF-κB pathway activation. Unfortunately, no advantages for the experimental arms were observed in any of the phase III trials that were looking for improvements in patients classified as ABC DLBCL using gene-expression profiling (34, 59, 60). A possible explanation of these negative results could be not only that treatments that have been explored are not optimal but also that the GCB and ABC subtypes defined at RNA level still comprise too heterogenous patients populations. The latter possibility is strongly sustained by recent studies that have looked at the genetic heterogeneity of DLBCL patients and have led to three novel subclassifications (19–21, 54).

A first classification identifies five clusters (C1-C5) (19) (**Table 1**). **C1** (18% of DLBCL) has cases with BCL6 chromosomal translocations, active NOTCH signaling (*NOTCH2* mutations, *SPEN* inactivation), active NF-κB pathway (*TNFAIP3* mutations or deletions, *BCL10* mutations), and immune escape mechanisms (inactivation of *CD70*, *CD58*, *FAS*, and structural variations of PD-L1 and PD-L2). **C2** (21% of DLBCL) is a mixture of GCB and ABC DLBCL, which share lesions in genes involved in the DNA damage response (*TP53* inactivation), cell cycle (inactivation of *CDKN2A* and *RB1*), PI3K/AKT signaling (*MIR17HG* amplifications), and apoptosis (*MCL1* gain or amplifications). **C3** (13% of all DLBCL) includes GCB-DLBCL with lesions affecting chromatin regulation (*EZH2* mutations, *KMT2D* mutations, *CREBBP* or *EP300* mutations or deletions), PI3K/AKT signaling (*PTEN* deletions or mutations, mTOR mutations, *MIR17HG* amplifications), apoptosis (*BCL2* chromosomal translocations), cell motility (*GNA13* mutations), and germinal center program (*MEF2B* or *IRF8* mutations). The GCB DLBCL **C4** (17% of all DLBCL) contains cases with genetic lesions affecting chromatin structure (mutations in linker and core histone genes), immune escape (*CD83*, *CD58*, and *CD70*), NF-κB pathway (mutations of *CARD11*, *NFKBIE*, and *NFKBIA*), BCR and PI3K signaling (mutations of *RHOA* and *SGK1*), cell motility (*GNA13* mutations), and RAS/JAK/STAT signaling (*BRAF* and *STAT3* mutations). The last one, **C5** (21% of all DLBCL) comprises ABC DLBCL cases with *BCL2* gains, concordant *MYD88* L265P/*CD79B* plus additional lesions such as gains of 3q, 19q13.42 and inactivation of *PRDM1*.

The second classification originally identified four subtypes (EZB, MCD, N1, and BN2) (20), which more recently have been extended to six (21) (**Table 2**). Cluster **EZB** (22% of DLBCL) resembles C3 and the genomic lesions of GCB DLBCL with lesions in genes coding for proteins involved in chromatin regulation (*EZH2* mutations, *KMT2D* mutations, *CREEBBP* or *EP300* mutations or deletions), apoptosis (BCL2 translocations), immune escape (*TNFRSF14* mutations or deletions), cell motility (*GNA13* mutations), JAK/STAT signaling (STAT6 mutations or amplifications, *SOCS1* mutations or deletions), PI3K/AKT signaling (*PTEN* deletions, mTOR mutations, and *MIR17HG*

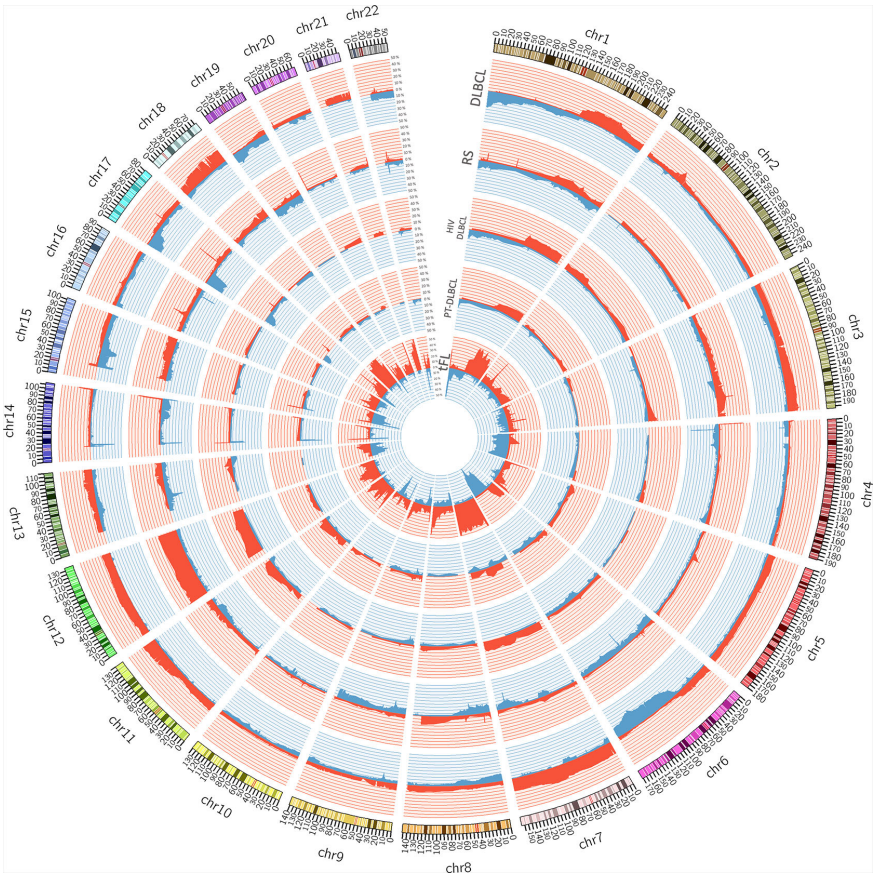

FIGURE 1 | Circos plot summarizing all the copy number changes observed in *de novo* DLBCL (n. = 22), Richter syndrome (RS, n. = 59), HIV-DLBCL (n. = 50), PT-DLBCL (n. = 44), and transformed FL (tFL, n. = 79). For each histology, the layers represent the frequency of copy number loss (blue) and gain (red). Data are obtained from published papers (28, 30, 33, 57, 58). The plot has been generated using Circos tool (v. 0.69) (53).

amplifications), immune escape (inactivation of TNFRSF14, *CIITA*, HLA-DMA), and *REL* amplifications. The **MCD** cluster (8% of DLBCL), similar to the C5, contains almost exclusively ABC-DLBCL with aberrant activation of the chronic BCR and NF-κB signaling (mutations of *MYD88*, *CD79A*, *CD79B*, and *CARD11*), impaired terminal B cell differentiation (*PRDM1* mutations or deletions, *SPIB* gains or amplifications), deregulated cell cycle (*CDKN2A/B* deletions), and immune escape (mutations or

TABLE 1 | DLBCL subtypes according to Chapuy et al. (19).

DLBCL subtype	COO	%	MUTATIONS	GENOMIC LESIONS
C1	ABC	18%	*BCL10, TNFAIP3, UBE2A, CD70, B2M, NOTCH2, TMEM30A, FAS, ZEB2, HLA-B, SPEN, PDCD1LG2/CD274*	GAINS: +5pFUSIONS AND TRANSLOCATIONS: 3q27 (*BCL6*), 9p24 (*PDCD1LG2/CD274*), 3q28 (*TP63*)
C2	ABC/ GCB	21%	*TP53*	GAINS: +1q23 (*MCL1*), +13q31 (*MIR17HG*), plus additional gross aberrations.LOSSES: -17p13 (*TP53*), -9p21 (*CDKN2A*), -13q14 (*RB1*), -1q42, plus additional gross aberrations.
C3	GCB	13%	*BCL2, CREBBP, EZH2, KMT2D, TNFRSF14, HVCN1, IRF8, GNA13, MEF2B, PTEN*	LOSSES: -10q23 (*PTEN*).FUSIONS AND TRANSLOCATIONS: 18q21 (*BCL2*).
C4	GCB	17%	*SGK1, HIST1H1E, NFKBIE, BRAF, CD83, NFKBIA, CD58, HIST1H2BC, STAT3, HIST1H1C, ZFP36L1, KLHL6, HIST1H1D, HIST1H1B, ETS1, TOX, HIST1H2AM, HIST1H2BK, RHOA, ACTB, LTB, SF3B1, CARD11, HIST1H2AC*	–
C5	ABC	21%	*CD79B, MYD88, ETV6, PIM1, TBL1XR1, GRHPR, ZC3H12A, HLA-A PRDM1, BTG1*	GAINS: +18q (*BCL2*, *MALT1*), +3q, +18p, +3p, +19q13.42, +19q.LOSSES : -17q25.1, -19p13.2, -6q21 (*PRDM1*).
C0*	ABC	4%	–	–

**unclassified.*

TABLE 2 | DLBCL subtypes according to Wright et al. (21).

DLBCL subtype	COO	%	MUTATIONS	GENOMIC LESIONS
MCD	ABC	9%	*MYD88* L265P, *CD79B, PIM1/2, HLA-A/B/C, BTG1/2, CDKN2A, ETV6, OSBPL10, TOX, MPEG1, SETD1B, KLHL14, TBL1XR1, GRHPR, PRDM1, CD58, TAP1, FOXC1, IRF4, VMP1, SLC1A5, DAZAP1, BCL11A, PPP1R9B, IL10RA, IL16, CHST2, ARID5B, WEE1, KLHL42 TNRC18*	GAINS: +18q21 (*BCL2*), +19q13 (*SPIB*, *SLC1A5*),+19p13 (*DAZAP1*).LOSSES: -6p21 (*HLA-A/B/C*, *TAP1*), -8q12 (TOX), -6q21 (*PRDM1*), - 1p13 (*CD58*), -9p21 (*CDKN2A*).FUSIONS AND TRANSLOCATIONS: 9p24 (*PDCD1LG2/CD274*).
EZB	GCB	20%	*EZH2, TNFRSF14, KMT2D, CREBBP, FAS, IRF8, EP300, MEF2B, CIITA, ARID1A, GNA13, STAT6, EBF1, GNAI2, C10orf12, BCL7A, HLA-DMB, S1PR2, MAP2K1, FBXO11*	GAINS: +2p16 (*REL*), chromosome 12p, + 12q13 (*STAT6*), chromosome 21, + 13q31 (*MIR17HG*)LOSSES: -10q23 (*PTEN*), -1p36 (*TNFRSF14*, *ARID1A*), -12q13 (*KMT2D*), -16p13 (*CREBBP*, CIITA), - 10q24 (*FAS*), -22q13 (*EP300*), -17q24 (*GNA13*), -5q33 (*EBF1*), -10q24 (*C10ORF12*), -15q22 (*MAP2K1*), -2p16 (*FBXO11*).FUSIONS AND TRANSLOCATIONS: 18q21 (*BCL2*), 16p13 (*CIITA*).
N1	ABC	2%	*NOTCH1, IRF2BP2, ID3, BCOR, EPB41, IKBKB, ALDH18A1*	GAINS: 4p.
BN2	ABC/ GCB	13%	*NOTCH2, TNFAIP3, DTX1, CD70, BCL10, UBE2A, TMEM30A, KLF2, SPEN, CCND3, NOL9, TP63, ETS1, HIST1H1D, PRKCB, HIST1H2BK, TRIP12, KLHL21, TRRAP, PABPC1*	GAINS: +1p12 (*NOTCH2*), +1p22 (*BCL10*), +16p12 (*PRKCB*).LOSSES: -6q23 (*TNFAIP3*), -6q14 (*TMEM30A*), -1p36 (*SPEN*), -3q28 (TP63).FUSIONS AND TRANSLOCATIONS: 3q27 (*BCL6*).
ST2	GCB	6%	*TET2, SGK1, DUSP2, ZFP36L1, ACTG1, ACTB, ITPKB, NFKBIA, STAT3, EIF4A2, JUNB, BCL2L1, DDX3X, SOCS1, CD83, P2RY8, RFTN1, RAC2, XBP1, SEC24C, MED16, PRRC2C, EDRF1, DOCK8, CLTC, ZNF516, WDR24, ZC3H12D*	LOSSES: -16p13 (*SOCS1*).
A53	ABC/ GCB	6%	*TP53, B2M, TP53BP1, TP73*	GAINS: +6p21 (*CNPY3*), +3q12 (*NFKBIZ*), plus additional gross aberrations.LOSSES: -17p13 (*TP53*), -15q21 (*B2M*), -15q15 (*TP53BP1*), -13q34 (*ING1*), 1p36 (*TP73*), plus additional gross aberrations.
unclassified	ABC/ GCB	37%	–	–

deletions of HLA-A, HLA-B, HLA-C, and *CD58*). The **N1** subtype (2% of DLBCL) mostly contains ABC DLBCL with Notch activation (*NOTCH1* mutations), NF-κB pathway (TNFAIP3 mutations or deletions), and impaired terminal B cell (lesions of *IRF4*, *ID3*, and *BCOR*). The **BN2** (15% of DLBCL), similar to C1, contains both GCB and ABC DLBCL and it is enriched of cases with Notch activation (*NOTCH2* mutations or amplifications, mutations of *DTX1* or *SPEN*), BCL6 translocations, NF-κB signaling (inactivation of *TNFAIP3* or *TN1P1* and gains or amplification of *PRKCB* and *BCL10*), immune escape (*CD70* inactivation), cell cycle (*CCND3* mutations), and cell migration (*CXCR5*). Since with this classification almost half of DLBCL cases did not fit in any defined subgroup (20), two additional subtypes have been proposed (ST2 and A53) (21). The **ST2** subtype (6% of DLBCL) is consists mostly of GCB DLBCL and is characterized by mutations in *TET2*, *SGK1* and JAK/STAT (*SOCS1* and *STAT3* mutations), and homing effectors (*GNA13* and *P2RY8*). The **A53** subtype is enriched of ABC DLBCL and is characterized by *TP53* mutations and deletions, with extensive aneuploidy, plus deletions of the *B2M* locus, amplifications of *CNPY3* (6p21), 6q losses (*TNFAIP3* and *PRDM1*), gain/amplification of 3q (*NFKBIZ*) and *BCL2* amplifications. Moreover, following the development of a double-hit gene expression signature identifying GCB-DLBCL patients with no evidence of a dual hit at FISH analysis but an outcome similar to the double-hit patients (36), the EZB group has been divided based on the presence (EZB-MYC+) or absence (EZB-MYC-) of a double hit (DHIT) signature (21).

Starting from a series of 928 cases that included also not *de novo* DLBCL and that were analyzed with a targeted panel of 293 genes, the last classification identifies five subgroups, with names based on their most common lesion (*MYD88*, *BCL2*, *SOCS1/SGK1*, *TET2/SGK1*, and *NOTCH2*), leaving 27% of cases unclassified (54) (**Table 3**). The **MYD88** cluster (16%) contains mostly ABC, and genes commonly mutated are *MYD88* (L265P),

TABLE 3 | DLBCL subtypes according to Lacy et al. (54).

DLBCL subtype	COO	%	MUTATIONS	GENOMIC LESIONS*
MYD88	ABC	16%	*MYD88, PIM1, CD79B, ETV6*	LOSSES: -9p21 (*CDKN2A*).
BCL2	GCB	19%	*EZH2, BLC2, CREBBP, TNFRSF14, KMT2D*	FUSIONS AND TRANSLOCATIONS: 18q21 (*BCL2*),
SOCS1/SGK1	GCB	12%	*SOCS1, CD83, SGK1, NFKBIA, HIST1H1E*	–
TET2/SGK1	GCB	11%	*TET2, BRAF, SGK1, KLHL6, ID3*	–
NOTCH2	ABC/GCB	15%	*NOTCH2, BLC10, TNFAIP3, CCND3, SPEN*	FUSIONS AND TRANSLOCATIONS: 3q27 (*BCL6*).
unclassified	ABC/GCB	27%	–	–

**the study performed targeted DNA sequencing on all the cases, while FISH analyses for BCL2 and BCL6 translocations were not done in all the cases done (54). CDKN2A data, based on sequencing data.*

PIM1, *CD79B*, and *ETV6* with also *CDKN2A* losses. The cluster overlaps with C5 and MCD from the other classifications (19, 21) and contains primary extranodal DLBCL (CNS; testis, breast). The **BCL2** cluster (19%) includes mostly GCB DLBCL and the majority of the cases that bear a *BCL2* translocation. It has high frequency of mutations of *EZH2*, *BCL2*, *CREBBP*, *TNFRSF14*, *KMT2D*, and *MEF2B*. The cluster overlaps with previously described C3 and EZB (19, 21) and contains most of the transformed FL included in the series. The **SOCS1/SGK1** group (12%) presents mutations of *SOCS1*, *CD83*, *SGK1*, *NFKBIA*, *HIST1H1E*, and *STAT3*. The **TET2/SGK1** cluster (11%) includes cases with mutations of *TET2*, *SGK1*, *KLHL6*, *ZFP36L1*, *BRAF*, *MAP2K1*, and *KRAS*. Both the SOCS1/SGK1 and the TET2/SGK1 clusters contain mostly GCB and overlap with the ST2 and C4 of the other classifications. Importantly, the SOCS1/SGK1 cluster also includes the PMBCL cases included in the study (*STAT3* and *SOCS1* mutations). The last cluster (**NOTCH2**, 15%) presents mutations of *NOTCH2*, *BCL10*, *TNFAIP3*, *CCND3*, *SPEN*, *TMEM30A*, *FAS*, and *CD70*, and cases with *BCL6* translocations. It has both GCB and ABC and it overlaps with the previously reported BN2 and C1 clusters (19, 21).

Although similar genetic features are picked up by the three classifications (19–21, 54), the final overlaps are only partial (**Table 4**), largely due to the approaches used by the Investigators to tackle the issue of DLBCL heterogeneity. However, the two large ABC and GCB subtypes have now been split in subgroups of cases bearing more similar genomic landscapes, and, thus, perhaps sharing more similar responses to targeted therapies (**Table 4**). New generation of clinical trials can now be designed to assess targeted agents, for example in addition to R-CHOP, in much better genetically defined subgroups of patients.

Interestingly, the genetics of the individual subtypes suggest that some DLBCL derive from the transformation of indolent lymphomas and/or that they follow specific pathogenetic mechanisms at least partially shared by other lymphoid neoplasms. These connections are evident for C5, MCD, MYD88 (primary extranodal DLBCL of the central nervous system or of the testis; transformed Waldenström macroglobulinemia), C3, EZB, BCL2 [follicular lymphoma (FL); transformed FL; Burkitt lymphoma), N1 [(NOTCH1 mutated chronic lymphocytic leukemia (CLL)], C2, BN2, NOTCH2 (transformed MZL), and ST2 (nodular lymphocyte-predominant Hodgkin lymphoma; T cell/histiocyte-rich large B cell lymphoma) (19–21, 54).

COPY NUMBER CHANGES AND TRANSFORMATION FROM INDOLENT LYMPHOMAS TO DLBCL

Copy number changes play important role in the transformation from indolent lymphomas to DLBCL and their presence can also be associated with a higher risk of transformation. Deregulation of MYC *via* DNA gains, amplifications or chromosomal translocation is the most frequent event occurring at the transformation from FL to DLBCL, followed by inactivation, mainly by DNA loss, of *CDKN2A/B*, of *B2M* (losses or mutations) and activating mutations of *P1M1* (28, 61). Transformed FL also have higher frequency of 3q and 11q gains than FL (28). Transformed FL and GCB DLBCL are phenotypically similar but their genomic profiles are not the same (28). Here, they present similar frequencies of 1p losses and 2p gains, but overall fewer occurrences of 13q gains (*MIR17HG*) or losses (*ING1*), as well of *PTEN* losses at 10q. Deletions of *TNFAIP3* and of *CDKN2A* are more common in transformed FCL than in GCB DLBCL (28).

A quite similar pattern is observed in the transformation from CLL to DLBCL (Richter syndrome) with the deletion at the *CDKN2A/B* locus as the most common acquired event (33, 37, 62). Despite the morphological appearance, as a whole, Richter syndrome has a CNV pattern that differs from *de novo* DLBCL, largely due to the under-representation of DNA gains and losses that are common in the latter disorder. Richter syndrome samples have a higher frequency of deletions at 7q31-q36 (still undefined role) and of the CLL related losses at 13q14.3 and 11q22.3 as well as trisomy 12 (**Figure 2**). Interestingly, copy number changes define two main subtypes of Richter syndrome (33). A first group (50% of Richter syndrome) bears *TP53* inactivation (by loss or by somatic mutations) and/or *CDKN2A* loss, alongside *MYC* gain/amplifications, 13q14.3 loss and additional lesions (33). A second group has almost exclusively trisomy 12 (33).

Regarding the risk of transformation to DLBCL, deletions at 1p35, 6q and copy neutral LOH at 16p have been associated with

TABLE 4 | Overlaps among DLBCL classifications and potential therapeutic interventions.

Cell of origin (46, 47)	DFCI/HMS * (19)	NCI** (20, 21),	HMRN*** (54)	Potential therapeutic interventions^
ABC	C5	MCD	MYD88 *	Lenalidomide; BTK inhibition; IRAK4 inhibition; BET inhibition; PI3K/mTOR inhibition; JAK/STAT inhibition; PKCβ inhibition BCL2 and BCL-XL inhibition
ABC	*	N1	*	Immune checkpoints; Notch1 inhibition
GCB	C3	EZB	BCL2	PI3K/mTOR inhibition; EZH2 inhibition; BCL2 inhibition; MYC inhibition; CREBBP inhibition
GCB	C4	*	SOCS1/ SGK1	JAK/STAT inhibition; BRAF/MEK1 inhibition
GCB	*	ST2	TET2/ SGK1	PI3K inhibition; JAK2 inhibition
GCB/ABC	C1	BN2	NOTCH2	BET inhibition; PI3K/mTOR inhibition; Lenalidomide; NF-kB inhibition; PKCβ inhibition; BCL2 inhibition; Notch2 inhibition
GCB/ABC	C2	A53	NEC	NF-kB inhibition; CDK inhibition

**Dana-Farber Cancer Institute/Harvard Medical School; **National Cancer Institute; ***Haematological Malignancy Research Network; ^(19–21).*

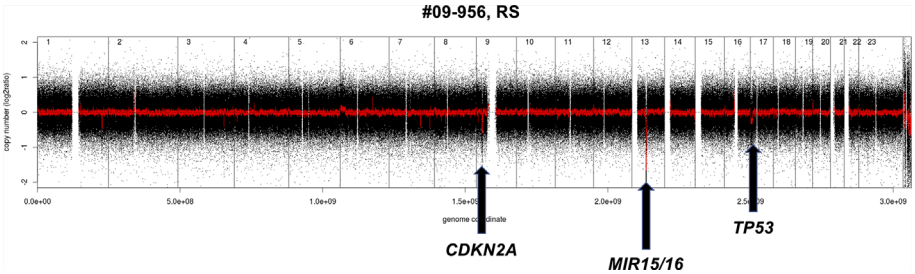

FIGURE 2 | Example of genomic profile of a RS case bearing the typical *CDKN2A* and *MIR15/16* deletion. Profiles obtained using the Affymetrix Genome-Wide Human SNP Array Version 6.0 [modified from (33)]. Black, raw copy number values; red, smoothed copy number values. X-axis, genomic mapping; Y-axis, log2 copy-number values.

higher risk of transformation to DLBCL in FL patients (32, 63). The presence of losses at 17p (*TP53*), 15q (*MGA*), and gains at 2p (*MYCN*, *REL*) and the lack of 13q14.3 deletions targeting *MIR15/MIR16* appeared linked with a higher risk of transformation to Richter syndrome from CLL (33).

COPY NUMBER CHANGES AND IMMUNODEFICIENCY-RELATED DLBCL

As there are differences in recurring CNV patterns between GCB and ABC DLBCL as well as between Richter syndrome and *de novo* DLBCL, a similar observation can be made when comparing the genomic profiles of DLBCL in immunocompetent individuals versus immunodeficiency related DLBCL. This became evident from studies comparing DLBCL obtained in persons with human immunodeficiency virus (HIV) infection in the pre-HAART (highly active antiretroviral therapy) (HIV-DLBCL) era, and in recipients of solid organ transplants (PT-DLBCL) with DLBCL from immunocompetent individuals, all analyzed with the same platform and data mining workflow (30, 57). First, a higher frequency of DNA breakages within fragile sites is seen in immunodeficiency related DLBCL than in immunocompetent cases, with perhaps a higher contribution of these changes to the etiology of the disease. Since viral DNA can insert in fragile sites, the immunodeficiency can expose the individuals to a multitude of viruses, which could infect B cells and integrate in the genome, preferentially at fragile sites (35, 38, 40, 52, 64–67).

Despite their phenotypic reminiscence of post-GC B-cells (29, 68), PT-DLBCL have a pattern of DNA gains and losses that is different from ABC DLBCL, lacking gains of 3q and 18q (*BCL2*, *NFATC1*) and losses of 6q (*PRDM1* and *TNFAIP3*) (57). Pre-HAART HIV-DLBCL show genomic profiles that are intermediate between ABC and GCB DLBCL, with more similarities towards the

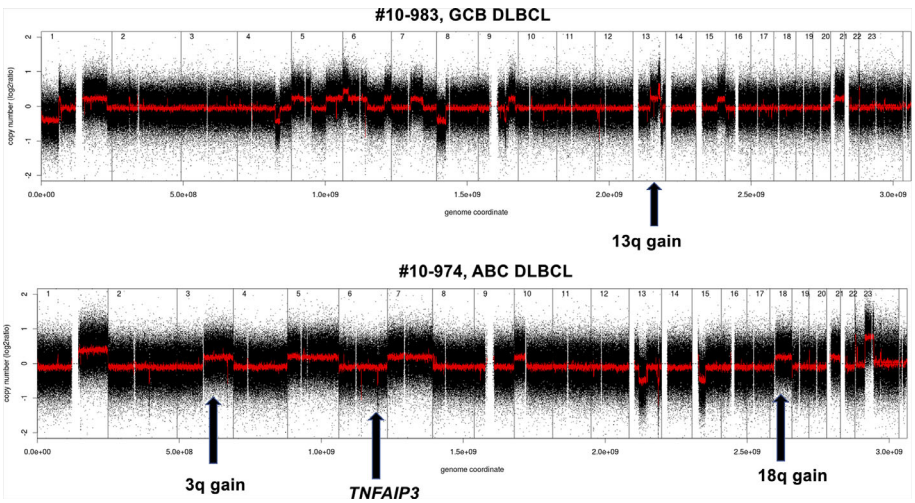

FIGURE 3 | Examples of genomic profile of two DLBCL cases bearing GCB (above) or ABC (below) lesions among others. Profiles obtained using the Affymetrix Genome-Wide Human SNP Array Version 6.0 [modified from (33)]. Black, raw copy number values; red, smoothed copy number values. X-axis, genomic mapping; Y-axis, log2 copy-number values.

latter. Indeed, HIV-DLBCL has GCB DLBCL lesions such as gains of 2p, 7q, and 12q, as well as losses of 1p, but it also carries 3q and 18q gains, commonly associated with ABC DLBC, and lacks the 10q deletions involving *PTEN* (30).

While gains of 1q, 11q and of chromosome 7 as well as 17p losses are present in both immunodeficiency related and immunocompetent DLBCL, deletions at 13q14 are usually absent (30, 57) suggesting a possible role in immune escape for the inactivation of *MIR15/MIR16* or of *RB1*, whose loci on 13q are frequently co-deleted in DLBCL (69). Interestingly, the loss of RB1 has been associated with T-cells exclusion in prostate cancer (70). Similarly, PT-DLBCL do not show copy neutral LOH (CN-LOH) affecting 6p, a common feature in different lymphomas including DLBCL and HIV-DLBCL. CN-LOH on 6p is believed to contribute to the silencing of the major histocompatibility complex (71) and DLBCL can indeed show absence or reduced expression of MHC class II proteins (27, 71–73). Importantly, the low MHC-II expression is associated with a decreased number of infiltrating T cells and reduced cytotoxic CD8+ T cells activation (27). Thus, it seems that the immune escape induced by the 6p copy neutral LOH is not required by PT-DLBCL but still needed by HIV-DLBCL. This could be due to the iatrogenic immunosuppression lowering both CD4+ and CD8+ T cells in the first lymphoma type while the viral infection causes a more pronounced loss of CD4+ than of cytotoxic CD8+ T-cells. Similarly, PT-DLBCL also have fewer B2M mutations—another immune escape mechanism—than immunocompetent DLBCL (74). It is also worth mentioning that among immunodeficiency related DLBCL the presence of Epstein Barr virus (EBV) is associated with a lower number of genomic lesions, both in terms of copy number changes and of somatic mutations (30, 57, 74, 75).

A global view of the different genomic profiles of DLBCL, Richter syndrome, immunodeficiency related DLBCL and transformed FL can be seen in **Figure 3**.

CONCLUSIONS

Data obtained in all these last years using genome wide technologies that allow for the molecular study of transcriptome profiles and of DNA changes (CNVs or somatic mutations) have led to building a much more precise framework to explain the heterogenous biology and clinical course of DLBCL cases. Although novel approaches such as the use of liquid biopsies are becoming increasingly feasible at least in the context of clinical trials, reproducible and commonly agreed genetic classification systems have to be defined. This is necessary to compare results from future individual clinical trials and to then transfer the findings to the right patients in the clinical practice. Indeed, the identification of group of patients with homogenous patterns of genetic lesions leading to the deregulation of specific pathways represents an opportunity to study novel agents in a more targeted approach than done so far, hopefully overcoming the disappointing results obtained trying to target the ABC DLBCL subtype defined based on gene expression profiling.

AUTHOR CONTRIBUTIONS

All authors participated to the design of the review, literature revision, manuscript writing, and final revision. All authors contributed to the article and approved the submitted version.

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